CD44 AND HYALURONAN BINDING IN MACROPHAGES AND DENDRITIC CELLS

by

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Abstract

Hyaluronan is a glycosaminoglycan present in pericellular and extracellular matrices. Hyaluronan production and binding are tightly regulated during an immune response yet its effects on immune cells, particularly macrophages and dendritic cells, are not fully understood. CD44 is the major surface receptor for hyaluronan, but not all CD44-expressing cells have the ability to bind hyaluronan. Here I demonstrate that hyaluronan binding is dynamically regulated during macrophage activation as well as dendritic cell differentiation, and CD44 provides macrophages with a selective advantage in the alveolar space that correlates with hyaluronan binding. Inflammatory macrophages activated with lipopolysaccharide plus interferon gamma bound higher levels hyaluronan compared to alternatively activated macrophages polarized with interleukin 4 by modifying chondroitin sulfation on CD44, and these macrophages differ in their ability to take up hyaluronan. In GM-CSF bone marrow-derived dendritic cell cultures, hyaluronan binding identified an immature population that was $CD11c^{+}$ MHC $II^{mid/low}$, highly proliferative and did not mature into MHC II^{high} cells. The removal of hyaluronan with hyaluronidase inhibited proliferation of the hyaluronan binding cells and promoted the maturation of dendritic cells. Furthermore, the hyaluronan binding cells had several macrophage characteristics and were retained in the alveolar space while the MHC II^{high}, low hyaluronan binding dendritic cells migrated to the lymph node upon inflammation. These results suggest a role for hyaluronan in cell proliferation and retention in the alveolar space. *In vivo*, peritoneal, splenic and lung interstitial macrophages bound low or no detectable levels of hyaluronan whereas alveolar macrophages constitutively bound high levels of hyaluronan. The alveolar space promoted hyaluronan binding, as instillation of peritoneal macrophages into the lung upregulated their ability to bind hyaluronan. During lung inflammation, the hyaluronan binding

population in the alveolar space was initially reduced and then restored upon resolution as a result of macrophage proliferation. In competition assays, CD44 provided hyaluronan binding macrophages with an advantage in the alveolar space. Collectively, this study reveals a novel role for CD44 in the maintenance of macrophages in the alveolar space and suggests that hyaluronan binding is induced at GM-CSF rich environments to help retain tissue macrophages at the site and promote their self-renewal.

Preface

I conducted all of the experimental research with the following exceptions:

- Brian Ruffell was responsible for Figures 3.4A and 3.4B.
- Sally Lee performed some experimental repeats for Figure 3.5A-C and was responsible for Figure 3.5D.
- Figure 4.1 was completed in collaboration with an undergraduate student, Kelsey Marshall under my mentorship. Kelsey was responsible for analyzing some of the surface markers expressed on GM-CSF bone marrow-derived dendritic cells by flow cytometry.
- Leslie Sanderson, an undergraduate student under my mentorship was responsible for the experiments involving the lung tissue in Figure 5.1A and 5.6B.
- Figure 5.8 was completed in collaboration with Sally Lee and Manisha Dosanjh. Sally Lee was responsible for irradiating the mice and performing bone marrow reconstitution. The tissues were collected together with Sally Lee and Manisha Dosanjh.

A version of Figures 3.2, 3.4 and 3.5 has been published. The research was originally published in the Journal of Biological Chemistry. Ruffell, B., **Poon, G.F.T.**, Lee, S.S.M., Brown, K.L., Tjew, S., Cooper, J., and P. Johnson. Differential use of chondroitin sulfate to regulate hyaluronan binding by receptor CD44 in inflammatory and interleukin 4-activated macrophages. *J Biol Chem* 2011; 286(22): 19179-90. © the American Society for Biochemistry and Molecular Biology. My work contributed to four figures in the publication, and I participated in the planning, discussion and writing of the original paper.

A version of Figures B1, B2 and B3 has been published: Brown, K.L.*, **Poon, G.F.T.***, Birkenhead, D., Pena, O.M., Falsafi, R., Dahlgren, C., Karlsson, A., Bylund, J., Hancock, R.E.W., and P. Johnson. (2011) Host defense peptide LL-37 selectively reduces proinflammatrory macrophage responses. *J Immunol*, 186(9): p. 5497-505. (* Both authors contributed equally to this work). *Copyright 2011. The American Association of Immunologists, Inc.* These figures were reproduced with the permission from the Journal of Immunology. My work contributed to four figures in the publication, and I participated in the experimental planning, discussion and the writing of the original and revised paper.

A version of Chapter 4 is now part of a manuscript in preparation and will be submitted for publication.

A version of Chapter 5 is now part of a manuscript in preparation and will be submitted for publication.

Animal experimentation was conducted in accordance with protocols approved by the University Animal Care Committee and Canadian Council of Animal Care guidelines. Project titles and the certificate numbers applicable to this project include:
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α	alpha
β	beta
γ	gamma
μ	micro
ζ	zeta

List of Abbreviations

BAL	bronchoalveolar lavage
BM	bone marrow
BMDC	bone marrow-derived dendritic cell
BMDM	bone marrow-derived macrophage
CCL	C-C chemokine ligand
CCR	C-C chemokine receptor
cDC	conventional DC
CS	chondroitin sulfate
CSF	colony stimulating factor
CX_3CR1	C-X3-C chemokine receptor 1
CHST1	carbohydrate (keratan sulfate Gal-6) sulfotransferase
CHST3	carbohydrate (chondroitin 6) sulfotransferase 3
CHST 7	carbohydrate (N-acetylglucosamine 6-O) sulfotransferase 7
DAMP	danger associated molecular pattern
DC	dendritic cell
E. coli	*Escherichia coli*
ECM	extracellular matrix
ELISA	enzyme-linked immunosorbent assay
FACS	fluorescence-activated cell sorting
FCS	fetal calf serum
Fl-HA	Fluorescein-conjugated HA
Flt3L	fms-like tyrosine kinase 3 ligand

Flt3	fms-like tyrosine kinase 3
GalNAc	galactose/ N-acetylgalactosamine
GalNacT	GalNAc transferase
GM-CSF	granulocyte and macrophage colony stimulating factor
HA	hyaluronan
HABP	hyaluronan binding protein
HAase	hyaluronidase
Has	hyaluronan synthase
HMW	high molecular weight
Hyal	hyaluronidase
IL	interleukin
IFNγ	interferon gamma
LAMP 1	lysosomal-associated membrane protein 1
LMW	low molecular weight
LPS	lipopolysaccharide
mAb	monoclonal antibody
MAMP	microbial associated molecular pattern
M-CSF	macrophage colony stimulating factor
MCP-1	monocyte chemoattractant protein-1
Mip	macrophage inflammatory protein
NK	natural killer
NO	nitric oxide
pDC	plasmacytoid DC

RANTES	regulated on activation normal T cell expressed and secreted
RBC	red blood cell
RT	room temperature
ROS	reactive oxygen species
TGF-β	transforming growth factor-beta
Th1	t helper 1
Tip-DC	TNFα and inducible nitric oxide synthase producing-DC
TNFα	tumor necrosis factor alpha

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Chapter 1: Introduction

1.1 The immune system

The immune system is a network of cells, tissues and organs that maintains tissue homeostasis by defending the body against pathogens and responding to tissue damage (Chen and Nunez, 2010; de Visser et al., 2006; Medzhitov, 2008). In most cases, the immune system will effectively distinguish between self and non-self, provide the appropriate response towards the antigen, and restore homeostasis, but sometimes, pathogens successfully evade the immune system and cause infection (Finlay and McFadden, 2006). Furthermore, defects in the immune system itself can also lead to various diseases. For example, the lack of immune response against cancerous cells results in tumor development (de Visser et al., 2006; Mantovani et al., 2008), excess tissue repair causes fibrosis (Wick et al., 2010), and inappropriate responses to self antigen leads to autoimmune disease (Medzhitov, 2008). Therefore, the immune system contributes to both health and disease.

1.1.1 The innate immune system

There are two major components to the immune system, the innate and the adaptive immune systems. Cells of the innate immune system include monocytes, macrophages, dendritic cells (DCs), natural killer (NK) cells, neutrophils, basophils, eosinophils and mast cells. Macrophages, DCs and mast cells are sentinel cells that are widely distributed in the tissues. They continuously monitor the environment for signs of pathogen invasion or tissue injury by recognizing microbial associated molecular patterns (MAMPs) or danger associated molecular patterns (DAMPs), respectively. When homeostasis is perturbed, they immediately secrete inflammatory mediators to control the situation and alert other immune cells, initiating an inflammatory response.

Neutrophils and monocytes are rapidly recruited to the site of infection from the blood stream. Neutrophils, monocytes and resident macrophages remove invading organisms by phagocytosis and release reactive oxygen species (ROS) that kill microbes, but also cause collateral damage by damaging host cells. While macrophages and neutrophils are effective in clearance of microbial infections, NK cells play a major role in the defense against virus-infected cells and tumor cells, and mast cells, basophils, and eosinophils are important in preventing parasitic infections. Although DCs are the most important antigen-presenting cells during primary immune responses, many of these other cell types also excel at processing antigen information from the site of inflammation, carrying antigens to the lymphoid organs and initiating adaptive immunity. Overall, the innate immune response is rapid, recognizes MAMPs and DAMPs, and informs the adaptive immune system about the infection.

1.1.2 The adaptive immune system

Inflammation mediated by the innate immune system is normally self-limiting and often sufficient to prevent infection. When this initial response is not sufficient, the adaptive immune system is activated. The adaptive immune system consists of T and B lymphocytes that express antigen-specific receptors. T and B lymphocytes that recognize the antigen transported to the lymphoid organ subsequently proliferate and differentiate into effectors cells. Activated B cells produce antibodies, soluble proteins that will bind to the antigen expressed on the pathogen and trigger the activation of the classical complement pathway, leading to cell lysis or phagocytosis by macrophages. Effector T cells can either directly kill the infected cells (cytotoxic T cells) or produce cytokines that promote the maturation of other immune cells like B cells or macrophages (helper T cells). Some of the activated T and B cells become memory cells that can

respond rapidly to the same antigen upon the next encounter. Thus, the adaptive immune response is specific and provides long-term protection.

1.2 Monocytes

1.2.1 Monocyte subsets

Monocytes are defined as circulating blood cells that can further differentiate into tissue macrophages and DCs (Auffray et al., 2009; Geissmann et al., 2010b; Serbina et al., 2008; Yona and Jung, 2009). Peripheral blood monocytes originate from the bone marrow (BM) where they develop from hematopoietic stem cells into different subsets of monocytes (Geissmann et al., 2010b; Serbina et al., 2008; Shi and Pamer, 2011; Yona and Jung, 2009). Recently, in addition to the BM and blood, the spleen is identified as another organ that holds monocytes. These splenic monocytes were recruited in greater number to ischemic myocardium compared to circulating monocytes and were indistinguishable from blood monocytes in terms of their surface markers, morphology and transcriptional profile (Swirski et al., 2009). Thus, monocytes are located in the BM, blood and spleen.

In mice, monocytes are $CD11b^{+}$ $F4/80^{low}$ $CD115^{+}$ and they are divided into two subsets based on their expression of Ly6C and CX_3CR1 (C-X3-C chemokine receptor 1) (Geissmann et al., 2010a; Geissmann et al., 2003; Serbina et al., 2008). The larger subset is $Ly6C^{high}$ CX_3CR1^{low} $CCR2^{+}$ (C-C chemokine receptor type 2), and the second population is $Ly6C^{low}$ CX_3CR1^{high} $CCR2^{-}$, these are present at an approximately 3:2 ratio in the blood (Landsman et al., 2009; Palframan et al., 2001). The $Ly6C^{high}$ monocytes are also known as inflammatory monocytes mainly because large numbers of these monocytes are recruited to infected organs in various models of infection such *Listeria monocytogenes, Mycobacteria tuberculosis* and

Toxoplasma gondii infections (Serbina et al., 2008; Shi and Pamer, 2011). Furthermore, adoptive transfer of peripheral blood monocytes showed that the monocytes recruited to the inflammatory site are predominately the Ly6C^{high} CX$_3$CR1^{low} monocytes, whereas the majority of Ly6C^{low} CX$_3$CR1^{high} remain in the blood (Geissmann et al., 2003; Palframan et al., 2001).

In contrast, in the absence of inflammation, adoptively transferred Ly6C^{high} monocytes are not detected in most organs, while Ly6C^{low} monocytes are recovered in the spleen, lung, liver and brain (Geissmann et al., 2003). The Ly6C^{low} monocytes crawl along the blood vessels and are in fact the first ones to enter the inflammatory site (Auffray et al., 2007). Hence, the Ly6C^{low} CX$_3$CR1^{high} monocytes are referred to as patrolling monocytes that constantly survey the environment (Auffray et al., 2007; Serbina et al., 2008). The expression of Ly6C and CX$_3$CR1 markers on monocyte subsets is not static. Ly6C^{high} monocytes labeled with fluorescent liposomes downregulates Ly6C expression and become Ly6C^{low} monocytes in the circulation (Sunderkotter et al., 2004). This demonstrates the ability of monocytes to change phenotype, and introduce the possibility that these monocyte subsets may be connected in some way. Perhaps changes in the expression of these surface markers represent monocyte maturation, where Ly6C^{high} CCR2^{+} CX$_3$CR1^{low} monocytes become Ly6C^{low} CCR2^{-} CX$_3$CR1^{high} monocytes before they differentiate into macrophages or DCs.

Human monocytes are also CD11b^{+} CD115^{+} and the two major subsets are distinguished by their CD14 and CD16 expression (Gordon and Taylor, 2005). The CD14^{high} CD16^{-} subset accounts for 90% of human blood monocytes and they resemble the murine Ly6C^{high} inflammatory monocytes in that they are CCR2^{+} CX$_3$CR1^{-}. The CD14^{+} CD16^{+} population is more similar to the Ly6C^{low} patrolling monocytes, with the phenotype CCR2^{-} CX$_3$CR1^{+}. In addition, there is a very small population with the intermediate phenotype CD14^{high} CD16^{+}

(Geissmann et al., 2003; Gordon and Taylor, 2005). The $CCR2^{+}$ CX_3CR1^{-} monocytes in both human ($CD14^{high}$ $CD16^{-}$) and mice ($Ly6C^{high}$) are rapidly recruited to sites of inflammation, but the direct comparison of physiological functions between monocyte subsets in human and mice is limited.

1.2.2 Monocyte function

Major functions of monocytes include, 1) patrolling the blood vessels for any signs of tissue distress and replenishing some tissue macrophages and DCs 2) phagocytosis of microbes and 3) the production of inflammatory mediators. Monocytes circulate in the blood to survey the environment and can replenish some tissue macrophage and DC populations (Hashimoto et al., 2011). The ability of monocytes to replenish tissue macrophages is often demonstrated during pathological settings, including microbial infections, atherosclerosis and cancer (Ingersoll et al., 2011; Qian et al., 2011; Serbina and Pamer, 2006). Monocytes can also replace lung macrophages after local macrophage depletion (Landsman and Jung, 2007), and several tissue macrophages are replaced by bone marrow cells following irradiation (Hashimoto et al., 2013; Landsman and Jung, 2007; Virolainen, 1968), suggesting monocytes also give rise to macrophages in the absence of pathology. However, emerging data suggest that monocytes do not always replenish macrophages during steady state, and macrophages instead repopulate by self-renewal. CCR2 is required for monocytes to exit the BM and enter circulation (Tsou et al., 2007). In $CCR2^{-/-}$ and wildtype parabiotic mice, only $CCR2^{+/+}$ monocytes from wildtype mice are able to replenished tissue macrophages in $CCR2^{-/-}$ mice. Although 70-90% of $CCR2^{+/+}$ monocytes were detected in the blood of parabiotic mice, the $CCR2^{+/+}$ monocytes did not replenish alveolar, peritoneal or splenic macrophages in the $CCR2^{-/-}$ host after 2 months. This

phenotype was maintained even after 1 year (Hashimoto et al., 2013). Langerhans cells and microglial cells both develop independently of monocytes and are repopulated by local proliferation (Ajami et al., 2007; Chorro et al., 2009). Therefore, monocytes have the capacity to maintain and replenish tissue macrophages in adult animals, but this process occurs under certain conditions, and it is currently unclear what determines macrophage repopulation by monocytes versus self-renewal.

Upon infection, monocytes are recruited to the site of inflammation and can give rise to pro-inflammatory cells that express DC markers, CD11c and MHC II, and are classified as monocyte-derived inflammatory DCs (Serbina and Pamer, 2006; Shi and Pamer, 2011). Following infection with *Listeria monocytogenes,* the $Ly6C^{high}$ monocytes are recruited from the BM and that the egress of the inflammatory monocyte from the bone into the blood is dependent on CCR2 expressed by the monocytes (Serbina and Pamer, 2006). Furthermore, these $Ly6C^{high}$ monocytes differentiate into inflammatory tumor necrosis factor alpha (TNFα) and nitric oxide (NO) producing $CD11b^+$ $CD11c^+$ MHC II^+ cells, called TNFα and inducible nitric oxide synthase producing-DC (Tip-DC) and this infiltration and differentiation of monocytes is important for bacterial clearance (Serbina and Pamer, 2006; Serbina et al., 2003). However, functional evidence demonstrating the ability of Tip-DCs to activate naïve T cells *in vivo* is lacking, and they have been suggested to be better described as activated monocytes or macrophages (Merad et al., 2013).

Monocytes isolated from the circulation can readily phagocytose various bacteria and are bactericidal (Cline and Swett, 1968; Steigbigel et al., 1974). Blood monocytes from hypertensive patients produce ROS, demonstrating the ability of monocytes to respond to infection or injury (Watanabe et al., 2006). In summary, monocytes not only have the ability to differentiate into

macrophages and Tip-DCs, they also respond to infections by eliminating microbes by phagocytosis and producing inflammatory mediators.

1.3 Macrophages

1.3.1 Introduction to macrophages

The term macrophage in Greek stands for "large eating cells" ("macro" means large, and "phago" for eating), which describes how they were first identified. These phagocytes were discovered by Metchnikoff as protectors against tissue invaders in 1883. By the 1890s, Metchnikoff recognized the importance of macrophages in host defense against infection from their ability to phagocytose and kill bacteria, and also their presence in wounds and their role in the clearance of unwanted cellular debris in the body (Tauber, 2003). Today, consistent with these earlier observations, macrophages are implicated in the initiation, progression, and the resolution of inflammation. Macrophages express receptors that allow them to respond to invading pathogens or damage by recognizing MAMPs or DAMPs (Soehnlein and Lindbom, 2010). Upon recognition, macrophages phagocytose substances and respond by producing inflammatory mediators that lead to the recruitment of other leukocytes and the killing of invading pathogens. Macrophages promote inflammation via the secretion of inflammatory cytokines such as TNFα, interleukin-1 alpha (IL-1α), IL-1 beta (IL-1β), and IL-6. They also produce pro-inflammatory chemokines such as monocyte chemoattractant protein -1 (MCP-1, also known as C-C chemokine ligand 2 (CCL2)), macrophage inflammatory protein-1 alpha (Mip-1α or CCL3), Mip-1β (or CCL4), and RANTES (regulated on activation normal T cell expressed and secreted or CCL5)), that can attract monocytes or neutrophils (Mantovani et al., 2004), and produce NO and ROS.

Macrophage functions can be influenced by neutrophils and their products. (Soehnlein and Lindbom, 2010). Neutrophils can convert macrophages to an anti-inflammatory phenotype and facilitate the resolution of inflammation. For example, neutrophils secrete peptides such as LL-37 to promote monocytes recruitment, and LL-37 also reduces the production of inflammatory cytokines by macrophages (Brown and Hancock, 2006). Furthermore, the presence of apoptotic neutrophils have been shown to down regulate the production of inflammatory cytokines IL-1 and TNFα and promote the secretion of the anti-inflammatory cytokine IL-10 by lipopolysaccharide (LPS) activated monocytes. Similarly, macrophages produce less inflammatory cytokines after they have taken up apoptotic neutrophils (Fadok et al., 1998; Soehnlein and Lindbom, 2010; Voll et al., 1997). To restore homeostasis, macrophages phagocytose apoptotic neutrophils and matrix debris, secrete anti-inflammatory molecules such as IL-10 to dampen the immune response, and produce transforming growth factor-beta (TGF-β) to promote tissue repair (Soehnlein and Lindbom, 2010). Therefore, although neutrophils are known to amplify the immune response, neutrophils can also signal macrophages to become anti-inflammatory and help transition towards the resolution of inflammation. Macrophages have several pro- and anti- inflammatory functions and impaired or uncontrolled macrophage function often leads to disease. For instance, excessive inflammatory responses from macrophages leads to chronic inflammation and contributes towards the pathogenesis of diseases such as atherosclerosis or Crohn's disease; however the lack of inflammation or too much of a healing response from macrophages can also have a negative effect on health, promoting diseases like cancer and fibrosis (Murray and Wynn, 2011).

1.3.2 M-CSF (CSF-1) in macrophage development and function

There are two growth factors that are important for the development of macrophages *in vivo*, macrophage colony stimulating factor (M-CSF) and granulocyte and macrophage colony stimulating factor (GM-CSF). The development of most tissue macrophages during homeostasis depends on M-CSF, also known as colony stimulating factor-1 (CSF-1). M-CSF is present throughout the body, particularly in the spleen red pulp and marginal zone, in the bone marrow, where it is produced by stromal fibroblasts and osteoblasts, and in the mammary gland (Ryan et al., 2001).

An extensive study by Cecchini *et al.* identified a deficiency in $F4/80^+$ macrophages in various organs in an osteopetrotic *(op/op)* mutant mouse strain (Yoshida et al., 1990) that lacks M-CSF due to a mutation in the M-CSF gene (Cecchini et al., 1994). Specifically, the development of macrophages in the liver, kidney and spleen is dependent on M-CSF, as demonstrated by the restoration of these population upon M-CSF treatment. Macrophages in the dermis, bladder, BM, and gut macrophages are partially dependent on M-CSF. In contrast, the development of $F4/80^+$ cells in the thymus and lymph node, as well as Langerhans cells (macrophages in the epidermis, but also known as DCs due to their ability to migrate to lymph nodes), are not deficient in the *op/op* mice, which suggests these macrophages develop independently of M-CSF (Cecchini et al., 1994).

Many of the phenotypes observed in the $CSF\text{-}1^{op/op}$ mice are also apparent in mice that lack the M-CSF receptor (Dai et al., 2002). Neutralizing antibodies against the M-CSF receptor deplete some macrophage populations, including $M\text{-}CSFR^+$ peritoneal macrophages and Langerhans cells, but do not deplete $M\text{-}CSFR^+$ cells in the lung. Consequently, inhibition of M-CSF receptor signaling do not diminish LPS-induced lung inflammation (MacDonald et al.,

2010). Interestingly, the M-CSF receptor blockage depletes the Langerhans cells in the skin whereas the lack of M-CSF did not, suggesting the role of additional M-CSFR ligands that may regulate the development of these cells. Indeed, IL-34 is recently identified as another M-CSFR ligand that regulates the development of Langerhans cells and microglia (macrophages in the brain) (Wang et al., 2012). Thus, M-CSF is essential for the development of several macrophage populations in the steady state and can promote the differentiation of BM cells as well as monocytes into macrophages *in vitro* (Hamilton, 2008). Overall, M-CSF is well known for its role in macrophage development, but not function.

1.3.3 GM-CSF (CSF-2) in macrophage development and function

GM-CSF (also known as CSF-2) is a hematopoietic growth factor that stimulates the development and maturation of myeloid cells such as granulocytes, macrophages and DCs (Burgess and Metcalf, 1980; Hamilton, 2008; Inaba et al., 1992; Lutz et al., 1999). GM-CSF does not have a role in the development of most macrophage populations but is essential for the development and function of alveolar macrophages (Guth et al., 2009; Paine et al., 2001; Shibata et al., 2001; Stanley et al., 1994). Normally, more than 90% of the cells collected from bronchoalveolar lavage (BAL) consist of $CD11c^+$ alveolar macrophages. In $GM\text{-}CSF^{-/-}$ mice, there are 4 times more cells in the BAL, but these cells are smaller and do not express CD11c to the same extent. In fact, most of the cells from $GM\text{-}CSF^{-/-}$ BAL are identified as $B220^+$ B and $CD4^+$ T lymphocytes, suggesting a lack of alveolar macrophage development in the absence of GM-CSF (Guth et al., 2009; Paine et al., 2001; Stanley et al., 1994). Functionally, cells collected from the BAL of $GM\text{-}CSF^{-/-}$ mice phagocytose fewer microbeads, produce less TNFα upon LPS stimulation compared to cells collected from $GM\text{-}CSF^{+/+}$ lung, (Paine et al., 2001), and *in vivo,*

GM-CSF deficient mice are also more susceptible to local infections (Hamilton and Anderson, 2004; Stanley et al., 1994). Together, these results confirm defective macrophage development in the absence of GM-CSF.

GM-CSF is a cytokine of many functions. GM-CSF promotes the progression of several diseases such as arthritis, asthma and experimental autoimmune encephalomyelitis, a murine model for multiple sclerosis. In other cases, GM-CSF treatment can suppress the development of type 1 diabetes and promote wound repair (Hamilton, 2008). GM-CSF has been categorized as a pro-inflammatory cytokine. The expression of GM-CSF is low in circulation during steady state but is enhanced upon inflammation, and it is expressed at higher levels in synovial fluids of patients with rheumatoid arthritis. Specifically, GM-CSF levels rapidly increase upon LPS stimulation in the lung and the addition of GM-CSF blocking antibody reduces inflammation (Bozinovski et al., 2004; Stanley et al., 1994).

GM-CSF is used to generate macrophages from BM cells (Fleetwood et al., 2007; Weisser et al., 2011) as well as peripheral blood monocytes *in vitro* (Sallusto and Lanzavecchia, 1994). Compared to M-CSF- derived macrophages, GM-CSF derived macrophages are considered more inflammatory as they express higher mRNA levels for *TNFα* and lower expression of *IL-10* (Fleetwood et al., 2007). Upon LPS stimulation, GM-CSF-macrophages produce higher levels of pro-inflammatory cytokines TNFα, IL-6, IL-12p70, IL-12p40 and IL-23 and less anti-inflammatory cytokine IL-10 than M-CSF derived macrophages (Fleetwood et al., 2007; Weisser et al., 2013). Interestingly, although macrophages differentiated in the presence of GM-CSF produce more inflammatory cytokines than those derived from M-CSF, they also express higher levels of arginase, a protein marker commonly used to identify healing

macrophages (Weisser et al., 2013). Therefore, GM-CSF derived macrophages are not only inflammatory, but may also play a role in promoting tissue repair.

1.3.4 Macrophage self-renewal by local proliferation

Although tissue macrophages are thought to be replenished by monocytes, particularly under inflammatory conditions, several studies now show that some macrophage populations can be maintained by local proliferation (Ajami et al., 2007; Geissmann et al., 2010b; Jenkins et al., 2011; Schulz et al., 2012). Despite different origins, microglia derived from yolk sac myeloid progenitors, and Langerhans cells derived from fetal liver monocytes are both self-renewing macrophages (Ajami et al., 2007; Chorro et al., 2009; Hoeffel et al., 2012). Peritoneal macrophages also proliferate locally upon parasite infection in an IL-4 dependent manner (Jenkins et al., 2011). Recently, a study by Hashimoto *et.al*, further illustrated the ability of lung macrophages to self-renew after local macrophage depletion, influenza infection and Poly (I:C) stimulation in the lung (Hashimoto et al., 2013). Following approximately 80% depletion of alveolar macrophages using CD169 diphtheria toxin receptor transgenic mice, macrophage repopulation occurred mainly by local proliferation and not from transplanted BM precursors. Specifically, the mRNA expression of *M-CSF* and *GM-CSF* but not *IL-4* increased in the lung following diphtheria toxin treatment. Furthermore, the expansion of alveolar macrophages is dependent on the expression of GM-CSF receptor (Hashimoto et al., 2013). After influenza infection and Poly (I:C) stimulation, there is a reduction in the number of macrophages in the lung along with monocyte infiltration, and the macrophage numbers recovered after 4 weeks independent of BM precursors or monocytes, suggesting the increase in macrophages occurred by local repopulation (Hashimoto et al., 2013). Interestingly, lung macrophages are replaced by

BM cells (Haniffa et al., 2009) and fetal liver cells (Hashimoto et al., 2013) subsequent to lethal irradiation. Therefore, lung macrophages can be replenished by both circulating precursor cells and by self-renewal. It appears that macrophages proliferate locally when there is a partial depletion. Macrophage self-renewal occurs after partial macrophages depletion in the lung (Hashimoto et al., 2013). A reduction in macrophage population was also observed following parasite infection in the pleural cavity (Jenkins et al., 2011) and influenza infection in the lung (Hashimoto et al., 2013) prior to macrophage proliferation. Overall, macrophages can self-renew through proliferation in the absence or presence of inflammation. However, signals that initiates macrophage local expansion and the mechanism by which IL-4 and GM-CSF regulates macrophage proliferation remains to be determined.

1.3.5 Macrophage heterogeneity and plasticity

In vivo, macrophages are present in various locations, including the bone, brain, lung, peritoneal cavity, liver and spleen (Gordon and Taylor, 2005; Hashimoto et al., 2011; Murray and Wynn, 2011; Pollard, 2009). There is considerable phenotypic and functional heterogeneity among tissue macrophages (Hashimoto et al., 2011; Hu et al., 2004; Hu et al., 2000; Laskin et al., 1988; Laskin et al., 2001; Morio et al., 2000). Although many macrophages are positive for F4/80 and CD11b, and negative for CD11c, as observed in peritoneal macrophages and splenic red pulp macrophages, alveolar macrophages express low levels of F4/80 and CD11b and are instead CD11c high, demonstrating the different phenotypes of tissue macrophages. Functionally, peritoneal macrophages are more efficient in phagocytosis of apoptotic thymocytes relative to alveolar macrophages (Hu et al., 2000). In another study, Kupffer cells in the liver also take up more propidium iodide-labeled *Staphylococcus aureus* than do alveolar macrophages, whereas

alveolar macrophages produce higher levels of superoxide anion and NO (Morio et al., 2000). Together, peritoneal macrophages and Kupffer cells are more efficient at phagocytosis compared to alveolar macrophages. Interestingly, both peritoneal macrophages and Kupffer cells depend on M-CSF for development, while alveolar macrophages require GM-CSF, suggesting that these growth factors are important regulators of macrophage function. However, M-CSF and GM-CSF alone do not completely explain the differences between tissue macrophages, because peritoneal macrophages are $CD11b^{high}$ but Kupffer cells express low levels of CD11b (Hashimoto et al., 2011).

The tissue environment can shape the phenotype of differentiated macrophages. After 7-14 days in the lung, $CD11c^{-}$ $CD11b^{+}$ peritoneal macrophages become $CD11c^{+}$ similar to alveolar macrophages, while still maintaining CD11b expression (Guth et al., 2009). Overall, M-CSF and GM-CSF likely contribute to tissue macrophage heterogeneity, but the additional differences among different subtypes of M-CSF derived macrophage suggest there must be other factors involved. To date, the exact signals that shape the functional and phenotypical heterogeneity in tissue macrophages are not well understood. These results also highlight the importance to extend *in vitro* findings to tissue macrophages, as macrophages differentiated *in vitro* lack environmental cues that shape their function, which may affect their response to a given treatment.

1.3.6 The M1 and M2 nomenclature

The M1and M2 macrophage nomenclature refers to macrophages that are polarized by either the T helper 1 (Th1) cytokine interferon gamma (IFNγ) in conjunction with microbial products such as LPS or cytokines like TNFα to become classically activated M1 macrophages, versus those

stimulated with the Th2 cytokines IL-4 and/or IL-13 to become alternatively activated M2 macrophages. M1 macrophages are efficient at the phagocytosis of bacteria and suppress tumor cell growth. These macrophages also rapidly produce pro-inflammatory cytokines and secrete ROS and NO upon stimulation by microbial products, and are therefore referred as inflammatory macrophages.

In contrast, M2 macrophages do not have inflammatory functions and are known to promote wound-healing and suppress T cell responses. The M2 macrophages are implicated diseases such as parasite infection, allergy, cancer, diabetes, and obesity, but due to the lack of specific markers and tools to deplete M2 macrophages without affecting other cell populations, their functional roles *in vivo* remains to be established (Gordon and Martinez, 2010; Mantovani et al., 2005; Mosser and Edwards, 2008; Van Dyken and Locksley, 2013). M2 macrophages exhibit arginase activity and contribute to the repair of the extracellular matrix (ECM), as arginase converts L-arginine into proline, a precursor for collagen synthesis as well as polyamines that promote fibroblast proliferation (Bronte and Zanovello, 2005; Gordon, 2003). *In vivo*, reduced arginase expression correlates with reduced muscle fiber regeneration, supporting the role of arginase in tissue repair (Ruffell et al., 2009). L-arginine is also required for T cell activation, and the depletion of L-arginine inhibits the expression of T cell receptor-associated CD3ζ polypeptide, and reduces T cell proliferation, thereby dampening the immune response. L-arginine has also been implicated in tumor progression (Bronte and Zanovello, 2005; Popovic et al., 2007; Van Dyken and Locksley, 2013). *In vivo*, M2 macrophage polarizing cytokine IL-4 promotes local macrophage proliferation in a Th2 parasite infection model (Jenkins et al., 2011). Therefore, IL-4 may help restore homeostasis following inflammation or infection by facilitating

the proliferation of non-inflammatory macrophages that downregulate T cell responses and promote healing and wound repair.

Another type of activated macrophages is the IL-10 producing regulatory macrophage, and because IL-10 suppresses the production of several inflammatory cytokines, these macrophages are anti-inflammatory (Mosser and Edwards, 2008). Because these macrophages are alternatively activated compared to the classically activated inflammatory macrophages, they are also referred to as a subtype of M2 macrophages, namely M2b macrophages. The activation of regulatory macrophages requires simultaneous stimulation with IFNγ plus LPS and additional reprogramming signals such as prostaglandin E2 or apoptotic cells, which induces the production IL-10, and similar to M2 macrophages, they express arginase. This demonstrates that other stimuli in addition to IL-4 can stimulate arginase activity, which is characteristic of M2 macrophages. While some include all non-classically activated macrophages with anti-inflammatory or healing functions as M2 macrophages, other researchers in the field have suggested to limit the M2 nomenclature to Th2 cytokines, IL-4 and/or IL-13 activated macrophages (Gordon and Martinez, 2010; Martinez et al., 2009). Macrophages *in vivo* are likely to show a continuum of these phenotypes as they encounter environmental stimuli, but M1 and M2 macrophages are useful *in vitro* generated phenotypes that can be used to examine macrophages that represent very distinct subtypes.

1.3.7 Macrophages and the host defense peptide LL-37

Defensins and anti-microbial peptides are a major component of the innate immune system and are produced mainly by epithelial cells in response to microbial challenge. They play an important role as a first line of defense at mucosal surfaces. LL-37 is a small cationic peptide

secreted by mucosal epithelial cells, neutrophils and macrophages, with both antimicrobial and immunomodulatory functions (Bowdish et al., 2005; Brown and Hancock, 2006). The concentration of LL-37 is elevated at sites of inflammation (Bals et al., 1999; Frohm et al., 1997; Ong et al., 2002; Schaller-Bals et al., 2002; Schauber and Gallo, 2008). LL-37 can kill microbes by directly interacting with the bacterial surface and causing membrane permeabilization (Zasloff, 2002). Accumulating evidence suggests that LL-37 has both pro- and anti-inflammatory functions, and that many of the LL-37 effects are mediated by monocytes and macrophages.

LL-37 plays a role in the recruitment of monocytes to inflammatory sites by inducing the production of MCP-1 by macrophages (Scott et al., 2002). *In vivo*, increased levels of MCP-1 were observed in the BAL of mice after receiving LL-37 in the lung (Scott et al., 2002). Although the presence of LL-37 augments production of the pro-inflammatory mediators such as IL-6, MCP -1 and MCP-3 by human blood monocytes in response to IL-1β and GM-CSF, the production of the same cytokines was reduced by LL-37 when the macrophages were activated by IFNγ, IL-4 or IL-12 (Yu et al., 2007). This demonstrates that the effect of LL-37 is context-dependent and specific to the cytokines acting on the cells. LL-37 has been shown to dramatically suppress the production of pro-inflammatory cytokines (TNFα, IL-12) induced by LPS, LPS and IFNγ, and lipoteichoic acid treatment of human monocytes, DCs and macrophages (Kandler et al., 2006; Mookherjee et al., 2006; Nijnik et al., 2009; Scott et al., 2000). Supporting these *in vitro* observations, treatment with LL-37 protects animals against sepsis caused by Gram-negative bacteria, as indicated by reduced levels of IL-6 and TNFα in the plasma (Cirioni et al., 2006).

LL-37 has also been implicated in wound healing (Carretero et al., 2008) and angiogenesis (Koczulla et al., 2003). In a wound healing model, animals given LL-37 showed significantly better re-epithelialization than to untreated animals (Carretero et al., 2008). LL-37 can limit inflammatory macrophage functions and dampen inflammation but its effect on M2 and tissue macrophages is not known. To be useful as an anti-inflammatory therapeutic, LL-37 would need to have specific effects on inflammatory macrophages without inhibiting the ability of macrophages to promote tissue repair.

1.4 Dendritic cells

1.4.1 Introduction to dendritic cells

DCs were first described by Ralph Steinman and Zanvil Cohn in 1973 as an adherent cell population found in the spleen, lymph node and Peyer's patches with "fine cell processes that constantly extend and retract like dendrites" (Steinman and Cohn, 1973). These cells were found to be potent stimulators in a mixed leukocyte reaction (Steinman and Witmer, 1978). DCs are found in the lymphoid organs, as well as non-lymphoid tissues. Functionally, DCs are specialized in their ability to process antigens from the tissue and then migrate to the peripheral lymphoid organs where they activate naïve T cells. DCs are largely defined by their high expression of CD11c and MHC II, but they also express a wide range of other cell surface markers. Based on their diverse phenotypes, locations and functions, there appears to be multiple subsets of DCs. Different DC populations are mainly divided into the conventional DCs (cDCs), plasmacytoid DCs (pDCs) and monocyte-derived inflammatory DCs. pDCs are morphologically, phenotypically and functionally distinct from other DCs, and provides immunity towards viral infections. cDCs most closely resemble the DCs identified by Steinman as they have a dendritic

phenotype, express MHC II and potently stimulate naïve T cells. At steady state, all non pDCs are categorized as cDCs, and the cDCs are further divided into several subtypes including, lymphoid DCs, non-lymphoid DCs, and Langerhans cells (Hashimoto et al., 2011; Merad et al., 2013; Satpathy et al., 2012; Shortman and Naik, 2007). There are two cytokines that are known to regulate the development of DC populations, the Fms-like tyrosine kinase 3 ligand (Flt3L) and GM-CSF.

1.4.2 Flt3L in dendritic cell development and function

Flt3L-deficient mice have dramatically reduced DC populations in the spleen, thymus and lymph nodes (McKenna et al., 2000). In addition, BM progenitors that lack the Flt3L receptor (Flt3) fail to develop into DCs but forced expression of Flt3 was able to rescue the phenotype (Onai et al., 2006). Therefore, Flt3L is required for DC development in lymphoid organs and regulates the development of pDCs and most cDCs *in vivo,* with the exception Langerhans cells (Merad et al., 2013; Onai et al., 2007). Flt3L is also used *in vitro* to promote the differentiation of BM cells into $CD11c^+$ MHC II^+ non-adherent DCs that are efficient at stimulating naïve T cell proliferation, which supports their role in DC differentiation (Brasel et al., 2000).

Normally, pDCs are round cells found in the blood and lymphoid organs. Upon activation with IL-13 and CD40 ligand, they mature into cells with dendrites, similar to classical DCs (Colonna et al., 2004). They are identified as being $CD11c^+$ $B220^+$ Siglec-H^+ in mice and $CD11c^-$ $B220^+$ $CD123^+$ in humans, and only express low levels of MHC II (Satpathy et al., 2012). pDCs express TLR-7 (Toll like receptor-7) and TLR-9, which allows them to recognize single-stranded RNA and unmethylated CpG-containing DNA, respectively. Functionally, they are characterized by their ability to secrete large amounts of type I interferon in response to viruses,

but are ineffective at activating T cells. In addition to the producing type I interferon that can activate NK cells, macrophages and dendritic cells and modulate the function of T and B cells, other cytokines produced by pDCs in response to TLR activation include pro-inflammatory cytokines TNFα, and IL-12, which are involved in Th1 cell differentiation. Therefore, pDC most likely protects against viral infections by secreting cytokines that can directly act on T cells, and indirectly activate T cell responses by promoting the maturation of DCs and activation of macrophages (Cervantes-Barragan et al., 2009; Colonna et al., 2004; Reizis et al., 2013).

Several subsets of DCs that express different sets of surface markers are included in the cDC category, and it has been suggested that the cDCs exist in two functional states, an immature state, and an activated state induced by microbial or other inflammatory stimuli (Satpathy et al., 2012). Immature DCs reside in peripheral tissues and are phagocytic with low MHC I and II expressions and are positive for CD11b and/or CD103 in addition to CD11c. The activated DCs process antigen, increase their expression of MHC II and the chemokine receptor CCR7, and migrate towards the T cell zone in lymphoid organs (Bachmann et al., 2006). Mature cDCs in the lymphoid organs are a heterogeneous population that can be further divided into subsets by their CD8 and CD4 expression. Generally, the $CD8^+$ DCs promote cytoxic T cell responses whereas the $CD8^-$ DCs promote CD4 helper T cell responses (Turley et al., 2010). Therefore, Flt3L is essential for the development of most DCs in lymphoid organs, which are responsible for initiating the adaptive immune response against both viral and microbial infections.

1.4.3 GM-CSF (CSF-2) in dendritic cell development and function

In contrast to Flt3L, GM-CSF is not required for the development of lymphoid DCs that reside in the lymphoid tissues as both GM-CSF and GM-CSF receptor-deficient mice only have minor defects in the resident DC populations in the spleen and lymph nodes (Hamilton and Achuthan, 2013; Vremec et al., 1997). Instead, GM-CSF is implicated in the development of non-lymphoid DCs and monocyte derived DC *in vivo* (Hamilton and Achuthan, 2013). A recent study by Greter *et al.* using GM-CSF receptor-, and GM-CSF- deficient mice showed that in the absence of GM-CSF signaling, there is reduced number of $CD103^+$ DCs in non-lymphoid organs such as the lung, skin and lamina propria (Greter et al., 2012), suggesting a role for GM-CSF in the development of cDCs in these organs. A reduce number of $CD103^+$ DCs in the lung and skin draining lymph nodes is also observed in both GM-CSF receptor-, and GM-CSF- deficient mice. Because $CD103^+$ DCs are known as migratory DCs, this reduction may be a result of reduced $CD103^+$ DCs in the tissue. However, GM-CSF could also have a role in regulating the maintenance of $CD103^+$ DCs in the lymphoid organs associated with the lung and skin. A role for GM-CSF in DC development is supported *in vitro*, as GM-CSF causes BM cells to differentiate into $CD11c^+$ MHC II^{mid} immature and MHC II^{high} mature DCs (Inaba et al., 1992; Lutz et al., 1999). Upon activation with MAMPs, there is an increase in the proportion of MHC II^{high} cells and up regulation of co-stimulatory molecules, and these GM-CSF BMDCs stimulate T cells effectively (Inaba et al., 1992; Lutz et al., 1999).

As well, GM-CSF along with IL-4 can differentiate blood monocytes into DCs (Xu et al., 2007). GM-CSF levels are elevated during inflammation, suggesting that GM-CSF plays a role in the development of monocyte-derived DCs, in particular the inflammatory Tip-DCs (Hamilton and Achuthan, 2013; Shortman and Naik, 2007). In an acute arthritis model, an increase in GM-

CSF in synovial fluid correlated with an increase in a $CD11c^+$ $CD11b^+$ MHC II^+ $Ly6C^+$ monocyte-like DC population that produced MCP-1 and the inflammatory cytokine IL-6. Importantly, this population is reduced in GM-$CSF^{-/-}$ mice (Campbell et al., 2007). The $CD11c^+$ $CD11b^+$ MHC II^+ phenotype of these DCs is similar to the Tip-DCs identified in the *Listeria monocytogenes* infection model (Serbina et al., 2003), supporting the idea that infection increases the production of monocyte-derived DCs. However, using wildtype and GM-CSF receptor-deficient mixed BM chimeric mice, the lack of GM-CSF receptor did not affect the development of Tip-DCs in several infection models including, influenza, *Streptococcus pneumoniae*, and *Salmonella* Typhimurium infection, as well as LPS-induced systemic inflammation. In addition, the number of $CD11c^+$ MHC II^+ $CD11b^+$ iNOS expressing inflammatory DCs are similar in wildtype and GM-CSF receptor-deficient mice infected with *Listeria monocytogenes*. In contrast to the earlier studies, these results suggest that GM-CSF signaling is not required for the generation of inflammatory Tip-DCs. One possible explanation for this difference between GM-CSF and GM-CSF receptor-deficient mice may be that GM-CSF can signal through other receptors, and the depletion of GM-CSF receptor Csf2rb2 does not inhibit all GM-CSF signaling. In summary, the role of GM-CSF in promoting inflammation upon infection by generating inflammatory monocyte-derived DCs is not clear, but GM-CSF is important in the development of DCs in mucosal tissues during homeostasis and these DCs are important for monitoring the environment. GM-CSF along with Flt3L regulate the development of DCs, however, it is currently not clear whether Flt3L- and GM-CSF-dependent DC development is specific to certain DC lineages or to the location of DCs.

1.5 Hyaluronan and CD44

1.5.1 Hyaluronan

Hyaluronan (HA) is a glycosaminoglycan that is distributed widely in the body as a major component of the extracellular matrix. The structure of HA consists of repeating units of the disaccharides, β (1, 4)-D-glucuronic acid and β (1, 3)-N-acetyl-D-glucosamine. HA is synthesized by hyaluronan synthases that are located on the inner surface of the plasma membrane and is extruded onto the cell surface or into the ECM. Therefore HA is part of both the extracellular and pericellular matrices.

During homeostasis, HA exists as a high molecular weight (HMW) species, containing up to 25,000 disaccharide units, and having a mass of 10^6 - 10^7 Da. HA binds large volumes of water and this generates a highly viscous and elastic environment. This property has linked HA to functions such as hydration, joint lubrication, and the maintenance of tissue structural integrity (Jiang et al., 2011; Laurent and Fraser, 1992; Laurent et al., 1996; Stern, 2005; Toole, 2009). HA plays an essential role in development, as mice deficient in hyaluronan synthase-2 exhibit embryonic lethality (Camenisch et al., 2000).

At sites of inflammation and tissue injury, HA fragments of different sizes are generated (Jiang et al., 2007; Taylor and Gallo, 2006). These HA fragments can act as DAMPs that induce sterile inflammation (Chen and Nunez, 2010). Accumulation of HA and HA fragments at the site of inflammation is correlated with disease severity, and their clearance is necessary for the resolution of inflammation (Teder et al., 2002). HA can also modulate the immune response, and these functions are largely dependent on its interaction with HA-binding proteins, such as CD44, the receptor for hyaluronan-mediated motility (also known as RHAMM), and TLRs (Jiang et al., 2011).

1.5.2 CD44

CD44 is the principal cell surface receptor for HA (Ponta et al., 2003; Sherman et al., 1994), and is implicated in multiple physiological and pathological immune responses (Cuff et al., 2001; Johnson and Ruffell, 2009; Ponta et al., 2003). CD44 consists of an extracellular domain that interacts with its major ligand HA, a stem structure within the extracellular region that includes motifs for posttranslational modifications as well as a site for the expression of variant exons, a transmembrane region that may interact with the lipid rafts to facilitate HA uptake and CD44 recycling (Thankamony and Knudson, 2006), and a cytoplasmic domain that binds to the ezrin, radixin and moesin proteins, which can associate with the cytoskeleton and regulate cell polarization and motility (Ponta et al., 2003). CD44 is expressed on most leukocytes including T and B lymphocytes, monocytes, macrophages, DCs, and eosinophils and has a role in facilitating cell adhesion (Lazaar et al., 1994), leukocyte recruitment (Cuff et al., 2001; DeGrendele et al., 1996; Hollingsworth et al., 2007; Leemans et al., 2003), modulating cell activation (Liang et al., 2007; Muto et al., 2009) and resolving inflammation (Teder et al., 2002).

Interestingly, the majority of immune cells that express CD44 do not bind HA in the absence of activating signals (Johnson and Ruffell, 2009).There are various mechanisms by which the ability of CD44 to bind HA is regulated, including increased CD44 expression, posttranslational modifications of CD44 such as glycosylation (English et al., 1998; Katoh et al., 1995; Skelton et al., 1998), glycosaminoglycan addition (Levesque and Haynes, 1999; Ruffell and Johnson, 2005), sialylation (Katoh et al., 1999), sulfation (Brown et al., 2001; Maiti et al., 1998) and the expression of different CD44 isoforms (Misra et al., 2011; Sleeman et al., 1996). Thus, CD44 is a heterogeneous protein that is subject to diverse modifications in response to

specific signals, and this allows CD44 to bind HA. CD44-null mice develop a normal immune system, with slightly reduced numbers of total number of spleen colony forming units cultured in the presence of erythropoietin, IL-3 or M-CSF compared to wildtype mice, demonstrating that CD44 does not have a major role in immune cell development (Schmits et al., 1997). However, when CD44-deficient animals are challenged in various disease models, there are significant differences between $CD44^{+/+}$ and $CD44^{-/-}$ mice (Johnson and Ruffell, 2009), which will be discussed in more detail in the following sections.

1.5.3 Hyaluronan in inflammation and disease

During an inflammatory response, components of the extracellular matrix are broken down by enzymes and reactive oxygen/nitrogen species (Duan and Kasper, 2011; Lu et al., 2011; Stern et al., 2007). HA fragments are often found at sites of inflammation. In the resolution phase of an inflammatory response, the production of full length HA and the clearance of the low molecular weight (LMW) HA both need to occur in order to restore HA homeostasis. The failure to remove excess HA correlates with more severe disease (Jiang et al., 2007, 2011; Lennon and Singleton, 2011). In animal models, HA accumulation (HMW and/or LMW) has been found at sites of atherosclerosis (Cuff et al., 2001), allograft rejection in the skin (Tesar et al., 2006), in the lungs of mice with bleomycin-induced pulmonary fibrosis (Teder et al., 2002), and in mice with LPS-induced lung inflammation (Hollingsworth et al., 2007). Similarly, patients with lung disorders such as chronic obstructive pulmonary disease (Dentener et al., 2005), bronchiolitis obliterans syndrome following lung transplant (Cuff et al., 2001), and idiopathic pulmonary arterial hypertension (Papakonstantinou et al., 2008), also have increased level of HA in the BAL fluid compared to healthy individuals. Tumor cells have also been shown to stimulate HA production

(Knudson et al., 1984), and the increased concentration of HA in tumors is positively correlated with malignancy (Toole, 2004). Therefore, HA production and degradation is tightly regulated and failure to remove excess HA and HA fragments correlates with disease severity.

HA functions are dependent on the size of HA. HMW HA is generally non-inflammatory and is more important for maintaining and restoring tissue structure (Stern et al., 2006). LMW HA on the other hand is thought to be pro-inflammatory, as LMW HA fragments can directly stimulate the production of inflammatory cytokines by DCs via their ability to interact with TLRs, independently of CD44 (Termeer et al., 2002; Termeer et al., 2000). In another study, intraperitoneal injection of HA increased the level of MIP-2 in the serum, and this was reduced in $CD44^{-/-}$ mice, suggesting CD44 is needed to respond to HA (Taylor et al., 2007). HA fragments (3-16 oligosaccharide units) but not large HA induce the proliferation of endothelial cells *in vitro* (West and Kumar, 1989). It was also shown that CD44 mediates HA-12 (12 oligosaccharide units)-induced endothelial cell proliferation and migration, as CD44 antibody inhibited both events (Trochon et al., 1996). Here, HA-induced cell proliferation and migration is dependent on the size of HA, as both un-degraded HA and HA-6 did not have an effect on endothelial cells (Trochon et al., 1996). Together, by interacting with different HA receptors, HA can promote inflammation by producing inflammatory cytokines, induce cell proliferation and migration, and this is often HA-size dependent.

The mechanism by which HA accumulation occurs may be due to dysregulation of HA synthesis, HA degradation, or HA uptake. In a rat model of monocrotaline-induced pulmonary hypertension, total HA in the lung is decreased early during the onset of disease, followed by an increase in HA as disease progresses (Ormiston et al., 2010). Correlating with the reduction in total HA was the enhanced expression of hyaluronidase-1 (*Hyal-1*) and the presence of LMW

HA at day 10 in the lung. By day 28, hyaluronan synthase-2 (*Has-2)* expression increased along with a downregulation of *Hyal-1*, and this may have led to increased HA synthesis and reduced degradation, resulting in excess HA accumulating in the lung (Ormiston et al., 2010). Interestingly, the expression of *Hyal-1* is increased in many different malignant tumors, correlating with elevated levels of total HA within tumors (Toole, 2004). Shar-Pei dogs are bred for wrinkles, and this trait correlates with increased periodic fever. Also, it was recently found that the wrinkles were rich in HA due to duplication in the *Has-2* gene, which correlates HA levels with periodic fever disorder (Olsson et al., 2011). Although the exact mechanism of HA clearance *in vivo* is still unclear, *ex vivo* alveolar macrophages can facilitate HA turnover during homeostasis via CD44, and this will be discussed in more detail in section 1.5.6. These results provide a strong connection between impairment in HA homeostasis and the development of inflammation and disease. However, there are still many unanswered question as to the signals that regulate HA synthesis and degradation, the specific cell types that mediate this process, and the mechanisms by which HA promotes disease.

1.5.4 CD44 in inflammation and disease

There is considerable evidence supporting a role for CD44 in regulating leukocyte trafficking. CD44 can interact with E-selectin, which is expressed on inflamed endothelia, and together with P-selectin mediates the recruitment of neutrophils to the site of inflammation (Katayama et al., 2005). Using 32D myeloid progenitor cells that were differentiated into neutrophils in the presence of granulocyte colony stimulating factor, it was demonstrated that the binding of these cells to E-selectin and P-selectin was dependent on N-linked and O linked glycosylation of CD44, respectively (Katayama et al., 2005). In addition, activated, HA-binding-competent CD44 on

effector T cells interacts with HA on endothelial cells to facilitate rolling and extravasation to the inflammatory site (DeGrendele et al., 1996). In an atherosclerosis model, macrophages were not recruited to the site of atherosclerotic lesions in $CD44^{-/-}$ mice (Cuff et al., 2001), supporting the idea that CD44 plays a role in cell adhesion and migration.

CD44 is also involved in the activation of leukocytes. For example, HA stimulates macrophages to produce inflammatory cytokines in a CD44 dependent manner (Taylor et al., 2007). Crosslinking of CD44 using antibodies against CD44 activates T cells to produce IL-2 and induces T cell proliferation (Foger et al., 2000). T cells are induced to proliferate after activation, and CD44 mediated HA binding identifies the most proliferative T cells, suggesting role for HA binding in activated T cells (Maeshima et al., 2011). Furthermore, the binding of HA to CD44 on re-activated T cells can induce cell death (Ruffell et al., 2011), demonstrating a different CD44 and HA role in T cells. Together, these results show that CD44 is involved in the activation of different immune cells and implicated in several functions.

The bleomycin-induced lung inflammation model clearly illustrated the importance of CD44 in the resolution of inflammation, as 75% of $CD44^{-/-}$ mice died after 2 weeks compared to $CD44^{+/+}$ mice, which all survived and resolved the inflammation. The $CD44^{-/-}$ animals exhibited an accumulation of macrophages, neutrophils and lymphocytes in the BAL and increased levels of HA and HA fragments in the lung, as well as a failure to take up apoptotic neutrophils and reduced generation of active TGF-β (Teder et al., 2002). *In vitro*, ligation of CD44 using a monoclonal antibody (mAb) against CD44 increased the ability of macrophages to phagocytose apoptotic neutrophils (Hart et al., 1997). These results implicate CD44 in various processes that are important for the resolution of inflammation. In summary, some of the roles of CD44 in

inflammation include its ability to: 1) to facilitate leukocyte trafficking 2) to regulate leukocyte activation and 3) in promotion of inflammation resolution.

1.5.5 Regulation of hyaluronan binding by monocytes

Although CD44 is expressed on many leukocytes, most of them normally do not bind HA. Leukocytes such as T cells (Lesley et al., 1994; Maeshima et al., 2011) and monocytes (Levesque and Haynes, 1997, 1999) exhibit increased HA binding upon activation. LPS as well as cytokines GM-CSF, IL-1α, IL-1β, IL-3 and TNFα, increase monocyte HA binding (Levesque and Haynes, 1997, 1999). In peripheral blood monocytes, HA binding is induced in the presence of TNFα (Brown et al., 2001; Levesque and Haynes, 1997), whereas IL-4 has been shown to inhibit TNFα-induced monocyte HA binding (Levesque and Haynes, 1997). TNFα-induced HA binding in a monocytic cell line and in human monocytes correlates with increased sulfation on CD44 (Brown et al., 2001; Maiti et al., 1998). This overall increase in sulfation is due to the additional sulfation of O- and N-linked carbohydrates in CD44. However there is also a decrease in the percent of sulfation due to chondroitin sulfate (CS) on CD44 (Delcommenne et al., 2002). Further work in our lab has identified CS addition to CD44 as a negative regulator of HA binding (Ruffell and Johnson, 2005). In summary, HA binding by monocytes is regulated by CD44 post-translational modifications, which can be induced by a number of cytokines. However, not all cytokines promote HA binding, as IL-4 downregulates, while TNFα induces HA binding. Interestingly, IL-4 stimulates the differentiation of M2 macrophages, whereas LPS and TNFα promotes the differentiation of inflammatory M1 macrophages, suggesting that HA binding may be differentially regulated in inflammatory versus healing macrophages. The ability and function of HA binding by macrophage subsets is not clear.

1.5.6 Regulation of macrophage function by CD44 and hyaluronan

A study by Cuff *et al*. demonstrated a role for CD44 in the recruitment of macrophages to the site of atherosclerotic lesions in apoE deficient mice (Cuff et al., 2001) and increased HA was found at the atherosclerotic lesions. The morphology and composition of the lesions are similar between $CD44^{+/+}$ and $CD44^{-/-}$ mice, with comparable proportion of macrophage-rich and matrix-rich fibrotic areas, suggesting that the lack of macrophage recruitment is not due to differences in the development of lesions. The role of CD44 in the recruitment of macrophages to HA rich lesions was clearly demonstrated, but whether this was facilitated by HA binding remains to be examined.

CD44 has also been shown to regulate LPS-induced lung inflammation through macrophages in the lung. CD44 negatively regulates inflammation, as the production of the inflammatory cytokines TNFα and MIP-2, as well as the number of macrophages, neutrophils and lymphocytes are increased in the BAL of CD44-deficeint mice, compared to wildtype mice. Specifically, expression of negative regulators involved in the LPS-TLR-4 signaling pathway such as A20, IL-1R-associated kinase-M and Toll-interacting protein are lower in $CD44^{-/-}$ macrophages, leading to a hyper immune response. These results show that CD44 promotes the expression of anti-inflammatory regulators macrophages, and thus negatively regulates LPS-induced inflammation (Liang et al., 2007). However, in another study that also used LPS to induce acute inflammation in the lungs, CD44 had the opposite effect, where fewer leukocytes were present in the BAL of mice that were deficient in CD44 (Hollingsworth et al., 2007). The explanation for these conflicting results is not apparent, but could be due to the different sources of LPS used, the amounts of LPS used, or different routes of administration in the two studies.

Specifically, Liang *et al*. stimulated mice with LPS from *Escherichia coli (E. coli)* 0127:B8 via intratracheal instillation at 1 mg/kg and Hollingsworth *et al.* used LPS from *E. coli* 0111.B4 and the concentration of LPS generated through aerosol was at 4-6 $\mu g/m^3$. Although these results are conflicting, both studies illustrate a role for CD44 in regulating LPS-induced inflammation. CD44 may mediate these effects by modifying LPS signaling pathways or via its ability to regulate leukocyte recruitment.

HA promotes the activation of macrophages and stimulates the production of inflammatory cytokines and chemokines. HA fragments upregulated the expression of genes encoding the inflammatory chemokines *MIP-1α*, *MIP-1β*, *RANTES*, and *MCP-1* in MH-S macrophages (mouse alveolar macrophage cell line) in a CD44-dependent manner (McKee et al., 1996). Similarly, small but not large HA induce the expression of IL-8 in inflammatory human alveolar macrophages collected from patients with idiopathic pulmonary fibrosis (McKee et al., 1996). Furthermore, HA purified from human umbilical cord stimulated the production of GM-CSF, TNFα, IL-1 and MIP-2 in MH-S macrophages (Taylor et al., 2007). In a Cytodex bead-induced sterile injury model, increased HA was accumulated at the site of injury. The addition of glycosaminoglycan purified from human wound fluid, which contains HA, induced MIP-2 production by macrophage. The effect of HA was distinct from LPS stimulation as HA induced IL-8 production was dependent on TLR-4 and MD2, while LPS required TLR-4, MD2 and CD14 (Taylor et al., 2007). Together, these studies show that HA fragments and HA collected from the site of injury can activate macrophages, and it is currently unclear whether this effect is specific to HA of a particular size.

Alveolar macrophages are the only macrophages known to constitutively bind high levels of HA and are able to internalize and degrade HA. HA binding and uptake by alveolar

macrophages requires the cell surface receptor CD44 (Culty et al., 1992), and the uptake of HA by macrophages is suggested to be important for the local turnover of HA (Underhill et al., 1993). CD44 plays a supportive role in the degradation of HA by Hyal-1 and Hyal-2 in a HEK-293 kidney cell line (Harada and Takahashi, 2007). However, whether macrophage CD44 regulates HA turnover during inflammation and the role of specific macrophage subsets is not known.

1.5.7 Functions of CD44 and hyaluronan in dendritic cells

LMW HA stimulates DC activation and promotes DC maturation. The addition of LMW HA of 4-14 oligosaccharides to GM-CSF bone marrow-derived dendritic cell (BMDC) cultures induces the production of inflammatory cytokines IL-1β, TNFα and IL-12 and upregulates the expression of the co-stimulatory molecules CD40, CD80 and CD86 in a TLR-4 dependent manner (Termeer et al., 2002; Termeer et al., 2000).

Inhibition of HA interaction with an HA blocking peptide modulates DC-driven, antigen specific T cell activation. Activated T cells were shown to express *Has-1 , Has-3* and *Hyal-2*, and DCs differentiated in the presence of GM-CSF also expressed *Has-1, 2, and 3* as well as *Hyal-1,2,3*, which are genes involved in the synthesis and degradation of HA. The addition of Pep-1, a positively charged peptide that blocks HA binding inhibited T cell activation with reduced proliferation, IL-2 and IFNγ production when T cells were activated by co-culture with antigen-loaded DCs, suggesting a role for HA in DC-mediated T cell activation. It was further shown that the addition of Pep-1 to T cells activated by mitogen, which does not involve DCs, was sufficient to inhibit T cell proliferation, suggesting that HA-DC interactions are not necessary to activate T cells (Mummert et al., 2002). Although HA can affect T cell activation in the absence of DCs, the role of HA in a DC-induced, antigen driven T cell activation remains

unknown. Furthermore, the observation that DCs express multiple hyaluronan synthase, *Has-1,2,3* and hyaluronidase, *Hyal-1,2,3* genes is intriguing, and it would be interesting to determine if DCs have a role in HA turnover.

HA has also been implicated in DC migration. *In vivo,* intravenous injection, subcutaneous injection and topical treatment with HA blocking peptide, Pep-1 prevented the infiltration of leukocytes and ear swelling in a hapten induced skin inflammation model. Langerhans cells can migrate out of the tissue into the lymph nodes upon inflammation, and after 24 h of hapten administration, the density and number of Langerhans cells were reduced at the site of stimulation. There was no reduction in Langerhans cells in the tissue when Pep-1 was given prior to hapten stimulation, suggesting that Pep-1 limits Langerhans cell migration out of the epidermis (Mummert et al., 2000). This supports a role for HA in modulating inflammation in the skin by regulating Langerhans cell trafficking out of the tissue, preventing their migration to the draining lymph node to activate T cells. However, it is also possible that Pep-1 prevents Langerhans cells from responding to the stimulus. As in macrophages, CD44 and HA are implicated in DC activation and migration, however the mechanism by which HA mediates this process remains to be established.

1.6 Research objective

HA accumulation correlates with a number of diseases, including lung fibrosis (Jiang et al., 2011; Teder et al., 2002), cancer (Misra et al., 2011; Toole, 2004), periodic fever (Olsson et al., 2011) and autoimmune diseases (Tesar et al., 2006). Growing evidence supports the need to maintain and restore HA homeostasis in order to resolve inflammation, but the mechanisms controlling HA fragmentation, accumulation and turnover, as well as how HA mediates disease progression,

are not well understood (Jiang et al., 2011; Lennon and Singleton, 2011; Misra et al., 2011; Stern et al., 2006). Macrophages and immature DCs are both localized to the site of inflammation where HA catabolism occurs. Both macrophages and DCs respond to LMW HA (McKee et al., 1996; Termeer et al., 2002; Termeer et al., 2000) and treatment with Pep-1, which blocks HA binding (Mummert et al., 2000), as well as the lack of the HA receptor CD44 (Cuff et al., 2001) affects the ability of these cells to move in and out of the tissue. This suggests that these cells sense HA concentration and size and respond to changes in HA. Cytokines such as TNFα, IL-4 and GM-CSF, which act on macrophages and DCs regulate HA binding by CD44 (Levesque and Haynes, 1997, 1999). Knowing the plasticity of macrophages and DCs and their roles in regulating the immune response, I hypothesize that macrophages and DCs activate CD44 upon stimulation in order to modulate their interaction with HA and either promote or dampen inflammation. In this thesis, I investigated the ability of macrophages and DCs to bind HA via CD44, and examined the function of HA binding by macrophages and DCs using *in vitro* and *in vivo* models.

The first part of this project examined the HA binding ability and function of HA binding in M1 and M2 macrophages. Macrophages have been implicated in the local turnover of HA during homeostasis (Culty et al., 1992), but whether the inflammatory or alternatively activated macrophages have distinct roles in HA turnover has not been determined. I hypothesize that M1 and M2 macrophages would have differential HA binding abilities that control HA turnover. Specifically, M1 macrophages would promote inflammation by generating HA fragments from HMW HA whereas M2 macrophages would be more efficient at the removal of LMW HA and thereby facilitate resolution.

Blocking HA interaction with Pep-1is suggested to limit Langerhans cell migration (Mummert et al., 2000), and the lack of CD44, the major HA receptor, prevents macrophage recruitment to atherosclerotic lesions (Cuff et al., 2001). These studies suggested a role for CD44 and HA in macrophage and DC trafficking at inflammatory sites. However, the role of HA binding in this process was not investigated. The second aim of this project was to examine the role of HA binding in DC maturation and migration and test the hypothesis that immature DCs bind HA resulting in DCs being retained in the tissue. In addition, I propose that HA binding is downregulated upon DC maturation in order to facilitate the migration of these cells out of the tissue.

To understand the physiological role of HA binding, we need to examine the role of HA binding by tissue macrophages *in vivo*. Alveolar macrophages are the only macrophages known to constitutively bind HA (Culty et al., 1992). In contrast, inflammatory cytokines can induce monocytes to bind HA, suggesting that HA binding may be regulated during the course of inflammation. However, the regulation of HA binding by monocytes and alveolar macrophages in an immune response has not been examined. The third aim of the project investigated the regulation and function of HA binding during LPS-induced lung inflammation. I hypothesize that HA binding facilitates the recruitment and retention of monocytes and alveolar macrophages in the alveolar space. Furthermore, the breakdown of HA will facilitate down regulation of HA binding at the peak of inflammation and allow the cells to regain motility.

Chapter 2: Materials and Methods

2.1 Materials

2.1.1 Mice

C57BL/6J mice expressing CD45.2, C57BL/6-Tg (CAG-EGFP) 10sb/J mice (from here on indicated as GFP^+ mice) with GFP under the control of beta-actin promoter, and B6.SJL-$Ptprc^a$ $Pepc^b$/ BoyJ mice (C57BL/6J mice expressing the congenic marker CD45.1, from here on indicated as BoyJ mice) were purchased from the Jackson laboratories. CD44 knockout mice ($CD44^{-/-}$) (Schmits et al., 1997) were backcrossed onto the C57BL/6J background for 6-9 generations. C57BL/6JxBoyJ heterozygotes express both CD45.1 and CD45.2 and were obtained by mating C57BL/6J and BoyJ mice. Animals were initially housed in the Wesbrook Animal Unit at the University of British Columbia, and later transferred to the Centre for Disease Modeling at the University of British Columbia. Mice used for experiments were between 6-15 weeks of age. All animal experiments were conducted in accordance with protocols approved by the University Animal Care Committee and Canadian Council of Animal Care guidelines.

2.1.2 Reagents

Recombinant mouse IL-4 and IFNγ were purchased from eBioscience. Recombinant mouse CCL3, CCL19 and TNFα were from R&D Systems. *E. coli* 0111:B4 LPS was from Invivogen. L-cell conditioned media (LCCM) is generated by L929 fibroblast cells (Earle et al., 1943) and is the source M-CSF. Tissue culture supernatant from J558L cells is the source of GM-CSF (Stockinger et al., 1996). Hyaluronan binding protein (HABP) conjugated to biotin and hyaluronidase from *Streptomyces hyaluronlyticus* nov. species were from Calbiochem. Hyaluronidases from bovine testes, *p*-nitrophenyl β-D-xylopyranoside (β-D xyloside), Greiss

reagent, *E. coli* 0111:B4 LPS (cat. L4391) and brefeldin A from *Penicillium brefeldianum* were purchased from Sigma-Aldrich. Dextran labeled with Alexa Fluor 647 was from Invitrogen. Fluorescein-conjugated HA (Fl-HA) was prepared as described (de Belder and Wik, 1975) using rooster comb hyaluronic acid sodium salt from Sigma Aldrich. HWM HA (1.68 x 10^6 Da) and LMW HA (28.6 kDa) were from Hyalose, L.L.C. and fluorescently conjugated with Alexa Fluor 647 by AbLab (UBC Antibody Lab, The Biomedical Research Centre, BC). The HA reagents from Hyalose L.L.C. were tested for endotoxin by the manufacturer, the HMW HA endotoxin level was <0.02 EU/mg and the LMW HA <0.002 EU/mg.

The LL-37 peptide (LLGDFFRKSKEKIGKEFKRIVQRIKDFFRNLVPRTES) was synthesized using F-moc chemistry at the Nucleic Acid/Protein Synthesis Unit, UBC. The peptide was resuspended in endotoxin-free water and was deemed free of endotoxin contamination as batches of the peptide were unable to stimulate TNFα production by myeloid cells.

2.1.3 Antibodies

The following antibodies against mouse antigens were used for flow cytometry and confocal microscopy: F4/80 (clone BM8), CD115 (clone Af598), CD11b (clone M1/70), CD11c (clone N418), Gr-1 (RB6-8C5), CD14 (clone Sa2-8), CD40 (HM40-3), CD80 (clone 16-10A1), CD86 (clone P03.1), MHC II (clone M5/114.15.2), CD44 (clone Im7), LAMP-1(clone ID48), Ly6C (clone AL21), Ly6G (clone 1A8), CCR5 (clone HMCCR5 (7A4)), CCR7 (clone 4B12), CD45.1 (clone A20), CD45.2 (clone 104), CD45 (clone I3/2), TNFα (clone MP6-XT66). Antibodies with the exception of CD44, CD45 and LAMP-1 were purchased from eBioscience or AbLab (UBC Antibody Lab, The Biomedical Research Centre, BC). Antibodies were conjugated to

fluorescein isothiocyanate (FITC), phycoerythrin (PE), phycoerythrin/ cyanine 7 (PE/Cy7), allophycocyanin (APC), pacific blue or biotin. Biotin conjugated antibodies were detected by fluorescent labeled streptavidin from eBioscience. Purified CD44 and CD45 mAb (American type Culture Collection, ATCC) were conjugated to Alexa Fluor 488, Alexa Fluor 647 or Pacific blue according to manufacturer's instructions (Molecular Probes). LAMP-1 was obtained from The Developmental Studies Hybridoma Bank at the University of Iowa. The source of Fc receptor blocking antibody 2.4G2 was HB-197 rat B cells (ATCC) and the tissue culture supernatant were used.

Antibodies used for Western blotting were, rat anti human/ mouse CD44 mAb, IM7.8.1 (ATCC#TIB-235), CD44 cytoplasmic domain rabbit antisera (J1WBB) and 3B3 (6-CS stub) mAb (Seikagaku America, East Falmouth, MA).

2.2 Methods

2.2.1 Differentiation and polarization of M-CSF derived M1 and M2 macrophages

BM cells were flushed from the femurs and tibias of mice, red blood cells (RBCs) were lysed with 0.84% ammonium chloride for 5 min at room temperature (RT) and then cells were plated at a density of 10 x 10^6 cells in a 100 x 15 mm petri dish (Falcon) in a 10 ml volume. On day 0, BM cells were cultured in DMEM (Invitrogen) supplemented with 20% fetal calf serum (FCS, from Invitrogen), 1 mM sodium pyruvate, 2 mM L-glutamine, 50 U/ml Penicillin/ Streptomycin (Invitrogen) and 5-7% LCCM (M-CSF). Media was removed on day 4, and replaced with fresh media containing 10% FCS and 5-7% LCCM. Adherent bone marrow-derived macrophages (BMDMs) were used for experiments on day 5. BMDMs were either cultured in BMDM media alone (unstimulated) or in the presence of 100 ng/ml *E. coli* 0111:B4 LPS (Invivogen, San Diego,

CA) and 50 ng/ml mouse recombinant IFNγ (eBioscience) to generate M1 BMDMs; 10 ng/ml of mouse recombinant IL-4 (eBioscience) was used to generate alternatively activated M2 BMDMs; or macrophages were activated with 20 ng/ml recombinant TNFα (R&D Systems). As specified in some experiments, BMDMs were also grown in the presence or absence of 2 mM β-D-xyloside (Sigma-Aldrich), a competitive inhibitor of glycosaminoglycan addition, to examine the effect of glycosaminoglycan modification on HA binding.

2.2.2 Generation of GM-CSF induced bone marrow-derived dendritic cells

BMDCs were prepared according to Lutz *et al.* (Lutz et al., 1999), with slight modifications. BM cells were isolated from the femurs and tibias of mice and the RBCs were lysed with 0.84% ammonium chloride and then cultured in RPMI (Invitrogen), containing 10% heat inactivated serum FCS (Invitrogen), 20 mM Hepes (Invitrogen), 1x non essential amino acid (Invitrogen), 55 μM 2 mercaptoethanol (Invitrogen), 50 U/ml Penicillin/ Streptomycin (Invitrogen), 1 mM sodium pyruvate, 2 mM L-glutamine and 2-4% GM-CSF. 2 x 10^5 cells/ml were cultured in a 10 ml volume in a 100 x 15 mm petri dish (Falcon), or in a 40 ml volume in 150 x 15 mm petri dish (Falcon). Media were changed on day 3 and day 6 (and day 9 when necessary) by removing half of the culturing media, spinning down the cells and resuspending in fresh media and GM-CSF. Non-adherent cells were harvested at the indicated time points, with cells for most BMDC studies collected on day 7 or 9. A typical yield on day 7 would be 5 x 10^6 cells and 25 x 10^6 cells for 10 ml and 40 ml culture respectively and approximately 70% $CD11c^+$ cells.

2.2.3 Cell isolation

Mice were euthanized by isoflurane overdose and alveolar cells were harvested from the BAL by catheterization of the trachea and washing three times, each with 1 ml PBS or PBS containing 1% BSA and 2 mM EDTA. After the BAL has been collected, a small opening in the left atrium was made using a needle, and then the lung was perfused by gently injecting 5 ml cold PBS containing 1% BSA and 2 mM EDTA into the right ventricle and allowing the fluid to flow out of the left atrium. The lung was minced in digest media containing RPMI and 0.7 mg/ml collagenase and 25 μg/ml DNAase and incubated at 37^{o}C for 10 min, followed by 30 min on the shaker at RT and then another 30 min at 37^{o}C. Lung tissues were filtered through a 70 μm strainer and the RBCs were lysed with 0.84% ammonium chloride for 5 min at RT to obtain single cell suspensions. The number of CD45^{+} leukocytes was obtained by multiplying the percentage of viable CD45^{+} (I3/2) with the total number of cells. To isolate splenocytes, the spleen was minced and pressed through a strainer in FACS buffer (1x PBS, 2 mM EDTA and 2% BSA), then treated with 0.84% ammonium chloride to lyse the RBCs. Peritoneal cells were harvested by peritoneal lavage with 5 ml of PBS. Cells were then spun down and treated with RBC lysis buffer for 5 min at RT. In some experiments, F4/80^{+} peritoneal macrophages were purified by positive selection through magnetic columns. Briefly, cells were resuspended in PBS with 0.5% BSA and 2 mM EDTA, then incubated with 2.4G2 for 20 min to block Fc receptors, then labeled with F4/80 biotinylated antibodies for 20 min. Cells were washed once, then incubated with anti-biotin magnetic beads (Miltenyi Biotec) in PBS with 0.5% BSA and 2 mM EDTA for another 20 min, and then passed through MACS LS columns (Miltenyi Biotec). The F480^{+} fraction was collected according to the manufacturer's instructions. Mediastinal lymph nodes were isolated and pressed through a 70 μm cell strainer in FACS buffer.

2.2.4 Dendritic cell stimulation in vitro

The effect of hyaluronidase on GM-CSF BMDC cultures was analyzed by collecting 2 x 10^5 non-adherent cells from a day 7 BMDC cultures and further incubating them with or without 20 U/ml *Streptomyces* or Testicular hyaluronidase in 100 µl BMDC media for 24 h. To study the effect of hyaluronidase on specific MHC II/HA binding subsets, non-adherent cells were harvested from a day 7 BMDC cultures and labeled with Fl-HA, CD11c, Gr1, MHC II mAb and propidium iodide (Sigma-Alrich) as described in 2.2.6 and different BMDC ($CD11c^+$ $Gr1^-$) populations were sorted by fluorescence-activated cell sorting (FACS). Equal numbers of cells (approximately 5 x 10^4) from each subset were plated in non tissue culture treated 96 well plates in the presence or absence of 20 U/ml *Streptomyces* or Testicular hyaluronidase or 100 ng/ml LPS (Invivogen) in 200 µl BMDC media and analyzed by flow cytometry at the specified time points. The percentage and number of MHC II^{high} cells in a given volume was obtained by running the samples on MACSQuant (Miltenyi Biotec) and then analyzed using FlowJo (Tree Star, Ashland, OR) software.

2.2.5 Macrophage stimulation with GM-CSF in vitro

2 x 10^5 M-CSF BMDMs collected on day 5 and 2 x 10^5 $F4/80^+$ peritoneal macrophages were cultured in 200 µl DMEM (Invitrogen) supplemented with 10% fetal calf serum (FCS), 1 mM sodium pyruvate, 2 mM L-glutamine, 50 U/ml Penicillin/ Streptomycin (Invitrogen), plated in non tissue culture treated 96 well plates and incubated in the presence or absence of 4% GM-CSF containing supernatant for 48 h and 72 h respectively. The phenotype of macrophages was analyzed by flow cytometry.

2.2.6 Flow cytometry

Cells (0.2-1 x 10^6) were incubated with 2.4G2 tissue culture supernatant for 20 min to block Fc receptors. Cells were washed with FACS buffer (1x PBS, 2 mM EDTA and 2% BSA), and then stained with mAb for 20 min protected from light. This step was repeated if secondary antibody staining was required. After washing, cells were resuspended in FACS buffer containing propidium iodide (Sigma-Alrich) to identify the non-viable cells. Cells were processed on LSR II (BD Biosciences) or MACSQuant (Miltenyi Biotec) and analyzed using FlowJo (Tree Star) software. Unstained cells were often used for negative gating, and this was confirmed by isotype controls as well as Fluorescence-Minus-One controls.

2.2.7 Measurement of hyaluronan and dextran uptake by flow cytometry

Day 5 BMDMs were stimulated in M1 and M2 polarizing agents for 24 h. 2 x 10^5 BMDMs were collected and cultured in 200 μl DMEM (Invitrogen) supplemented with 10% FCS, 1 mM sodium pyruvate, 2 mM L-glutamine, 50 U/ml Penicillin/ Streptomycin (Invitrogen), plated in non tissue culture treated 96 well plates with or without 5 μg/ml of Fl-HA (HA from Sigma Aldrich), Alexa Fluor 649 conjugated HMW HA (Hyalose L.L.C.), Alexa Fluor 649 conjugated LMW HA (Hyalose L.L.C.) or 20 μg/ml Alexa Fluor 647 conjugated dextran (Invitrogen) for another 24 h at 37^{o}C with 5% CO_2. Surface HA was removed by treating with 1 U/ml *Streptomyces* hyaluronidase in 100 μl FACs buffer for 5 min on ice, and the amount of intracellular HA and dextran was assessed by flow cytometry.

2 x 10^5 day 9 BMDCs were cultured in 200 μl DC media without GM-CSF and were further cultured with 10 μg/ml Alexa Fluor 647 conjugated dextran (Invitrogen) for 45 min at

37°C with 5% CO_2. Then cells were labeled with Fl-HA and mAbs on ice to determine the HA binding ability and phenotype of cells that take up dextran.

2.2.8 TNFα ELISA

After 24 h stimulation, tissue culture supernatants from M1 and M2 BMDMs were collected and TNFα production was measured by a standard, sandwich enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's directions (eBioscience).

2.2.9 Nitric oxide assay

Supernatant from M1 and M2 BMDMs stimulated for 24 h were analyzed for the amount of NO by adding equal volumes of tissue culture supernatant with Greiss reagent (Sigma-Aldrich) followed by 15 min incubation in the dark. Measurements were obtained by comparing the optical density at 540 nm against a sodium nitrite standard curve, ranging from 0.55 to 70 μM. Optical density was measured using a SpectraMax 190 (Sunnyvale, CA) plate reader.

2.2.10 Arginase assay

Following polarization, M1 and M2 macrophages were lysed at 5 x 10^5 cells/ml in a solution of 20 mM Tris pH 7.5, 150 mM NaCl, 2 mM EDTA, 0.1% Trition-X-100 containing 1x protease inhibitor cocktail for 30 min at RT. Lysates (100 μl) were mixed with an equal volume of 10 mM Tris-HCl pH 7.5 and 1/10 volume of 10 mM $MgCl_2$, heated at 56°C for 10 min then incubated with 100 μl of 0.5 M L-arginine at 37°C for 1 h. 800 μl of acid solution (7:3:1, H_2O:H_3PO_4:H_2SO_4) and 40 μl of 9% α-isonitrosopropiophenone (ISPF; in 100% EtOH) were added to the samples as well as a dilution series of urea for the standards. Samples and standards

were heated at 95°C for 30 min, placed in the dark (10 min), transferred (200 μl) to a 96-well flat bottom plate and the optical density was read at 540 nm using a SpectraMax 190 (Sunnyvale, CA) plate reader.

2.2.11 Analysis of chondroitin sulfate on CD44 by Western blotting

Immunoprecipitation, sulfate labeling and Western blotting of CD44 were performed as described (Ruffell and Johnson, 2005). Briefly, cells were cultured for the last 2 days in $Na_2[^{35}SO_4]$ during a 3 day incubation with M1 and M2 polarizing media, TNFα and/or β-D-xyloside. Cells were lysed, incubated with IM7-coupled beads and then immunoprecipitated CD44 was resolved on a 7.5% SDS-polyacrylamide gel and transferred to a polyvinylidene difluoride membrane (Millipore, Mississauga, ON). Membranes were exposed to Kodak BioMax MR film (Interscience, Markham, ON) at -80°C for 7 to 10 days. To determine relative CD44 levels, membranes were labeled with Ab against CD44 (J1WBB) and HRP-goat anti rabbit Ab. To detect chondroitin sulfate, immunoprecipitated CD44 from ~2.5 x 10^7 cells was digested with chondroitinase ABC (Seikagaku, America) and blotted with the 3B3 mAb, which recognizes the 6-sulfated chondroitin sulfate stub. Following incubation with HRP-goat anti mouse Ab, membranes were developed with enhanced chemiluminescence (ECL, Amersham Biosciences) according to the manufacturer's instructions.

2.2.12 Semi-quantitative PCR

The primers and annealing temperatures for the mouse carbohydrate transferases, chondroitin sulfate N-acetyl galactosamine transferases 1 and 2 (CSGalNAcT1 and CSGalNAcT2), N-Acetyl galactosamine 6-sulfotransferase (CHST3, also known as C6ST1), N-Acetyl

glucosamine/galactosamine 6-sulfotransferase (CHST7, also known as C6ST2), keratan sulfate galactose 6-sulfotransferase (CHST1, also known as KSGal6ST) and GAPDH are as follows: CSGalNAcT1, 56^{o}C, 5'-AGAAGAAATAAATGAAGTCAAAGGAATAC-3' and 5'-GAAGTAGATGTCCACATCACAG-3' which formed an 181 bp fragment and CSGalNAcT2, 40^{o}C, 5'-CCTAGAATCTGTCACCAGT-3' and 5'-GTTAAGGAATTCGGCTGAGAAATA-3' which formed an 172 bp fragment (Izumikawa et al., 2011); CHST3, 60^{o}C, 5'-GGACCTTGTACACAGCCTAAAGATTCG-3' and 5'-CTCGGACAGCCACTTCTTCCA-3' formed a 928 bp fragment; CHST7, 60^{o}C, 5'-ACCCAGGAAAAGCAACACATCTATG-3' and 5'-GGTTAAGAAGAAATCAGCGCGTGG-3' formed a 735 bp fragment; CHST1, 60^{o}C, 5'- AGTACACAGCCATCCGCACTT-3' and 5'- TGTGCCACGTGACTGTCCA-3' formed a 934 bp fragment, and GAPDH, 60^{o}C, 5'-ACCACAGTCCATGCCATCAC-3' and 5'-CACCACCCTGTTGCTGTAGCC-3' which formed a 450 bp fragment. mRNA expression levels were measured using real time PCR. Briefly, 250 ng of total RNA from unstimulated BMDMs, or BMDMs stimulated with LPS plus IFNγ or IL-4 for 24 h using TRIzol Reagent (Invitrogen), was reverse transcribed with iScript (BioRad, Hercules, CA), according to the manufacturers' instructions. An aliquot of the cDNA was subjected to PCR (25-35 cycles) with 0.5 U Platinum Taq polymerase (Invitrogen) in 20 μl. The PCR product was electrophoresed in 1.2% agarose gel, stained with SYBR Safe (Invitrogen) and visualized under ultraviolet light.

2.2.13 Confocal microscopy

C57BL/6J and CD44$^{-/-}$ BM cells were differentiated in M-CSF for 5 days, and then stimulated with M1 or M2 polarizing agents for 24 h. 1 x 10^5 cells were transferred to a 8 well chamber slide (Lab-Tek, Naperville, IL) and cultured in 200 μl of BMDM media (without M-CSF) with

or without 5 μg/ml of Fl-HA (HA from Sigma-Aldrich) or 20 μg/ml Alexa Fluor 647 conjugated dextran for 24 h at 37^{o}C with 5% CO_2, protected from light. After 24 h, cells were washed once with PBS, by aspirating the media and adding 400 μl PBS to the wells. Cells were treated with 1 U/ml *Streptomyces* hyaluronidase for 5 min on ice to remove surface bound HA and then fixed with 400 μl of 4% paraformaldehyde for 20 min at RT. Cells were washed twice with PBS and 200 μl of 2.4G2 Ab was added to each well to block Fc receptors and incubated for 20 min RT. Cells were then incubated with CD44-Alexa Fluor 568 or LAMP-1 mAb for 20 min RT. Cells stained with LAMP-1 mAb were further stained with goat anti rat secondary antibody for 20 min RT. Cells were mounted with ProLong Gold Antifade Reagent with DAPI (Molecular Probes). Confocal images were captured using Olympus Fluoview FV1000 Laser Scanning Confocal Microscope (Olympus).

2.2.14 Measurement of intracellular TNFα in alveolar or peritoneal macrophages

BAL and peritoneal lavage cells were resuspended and cultured in DMEM/10% FCS. Alveolar cells and peritoneal cells, were cultured at 5 x 10^4/well and 1 x 10^5/well, respectively, in a 96-well plate (Corning Costar, Corning, NY), in a final volume of 200 μl. Cells from each mouse were left unstimulated or, stimulated with 100 ng/ml of LPS (cat. L4391, Sigma-Aldrich), 20 μg/ml LL-37, or a combination of LPS and LL-37 for 2 h.

Peritoneal cells were stimulated *in vitro* for 2 h as described above in the presence of 10 μg/ml brefeldin A (Sigma-Aldrich) to suppress secretion and allow for the accumulation of intracellular TNFα. Alveolar cells after *in vitro* or *in vivo* treatment (see below) did not require brefeldin A treatment to detect intracellular TNFα. Alveolar (5 x 10^4) or peritoneal cells (1 x 10^5) were incubated with an Fc receptor blocking mAb, 2.4G2, for 20 min on ice, then labeled with

CD11c or F/480 mAb for 20 min on ice. Cells were fixed with 1% paraformaldehyde (Canemco and Marivac, Quebec, Canada) and then permeabilized with 0.1% saponin (Calbiochem, La Jolla, CA) for 10 min. Intracellular labeling was performed by incubating with anti-TNFα mAb or PE conjugated IgG_1 isotype control Ab diluted in 0.1% saponin buffer for 45 min then washed. Flow cytometry was performed on an LSR II flow cytometer (BD Biosciences) and data analyzed using FlowJo (Tree Star) software. Intracellular levels of TNFα were determined on $F4/80^+$ peritoneal macrophages and $CD11c^+$ alveolar macrophages. Typically, 50**%** of peritoneal cells were $F4/80^+$ macrophages (the other major cell population was B cells). In the BAL, 80–90% of the cells were $CD11c^+$ alveolar macrophages.

2.2.15 Migration assay

Non-adherent cells were collected from day 8 GM-CSF BMDC cultures, and were resuspended in fresh BMDC media without GM-CSF in the presence or absence of 40 ng/ml CCL3 (R&D systems) or 40 ng/ml CCL19 (R&D systems). The transwell migration assays were performed using 24- well plates with 6.5 mm diameter wells and inserts with 5 μm sized pores (Costar). 500 μl of media with or without the chemoattractant were placed in the bottom of the well, and 5 x 10^5 cells were placed onto the insert in 100 μl volume and the cells were incubated at 37^oC with 5% CO_2 for 2 h for experiments involving CCL19, and 3 h or 24 h for assays involving CCL3. Cells that have migrated out of the porous inserts into the bottom of the well were analyzed for their phenotype and counted using the MACSQuant (Miltenyi Biotec). In some wells, 5 x 10^5 cells in 100 μl media were placed into the bottom of the well with 500 μl of media without chemokines and were treated equally as the other conditions; cells collected from these wells

represent the total number of cells in this assay. % migration = number of cells migrated in the presence or absence of chemokine/ total number of cells in this assay x100.

2.2.16 In vivo LPS-induced lung inflammation

To determine the effect of LL-37 in LPS-induced lung inflammation, the same amount of LPS used to study *ex vivo* and *in vitro* macrophages (see above) was instilled into the lung. Anesthetized mice were injected by intratracheal instillation with 50 μl of PBS, 50 μl of PBS containing 100 ng LPS (Sigma-Aldrich, cat. L4391) or 60 μl of PBS containing 100 ng LPS and 20 μg LL-37. After 1 h, mice were euthanized by isoflurane over-dose. The supernatant from the first 1 ml of BAL (collected with PBS) was assayed for TNFα by ELISA. The cells pooled from 3 ml of BAL were used for intracellular TNFα analysis by flow cytometry.

In all other studies, animals were treated with 25 μg of LPS (Sigma-Aldrich, cat. L4391) in 50 μl PBS. The BAL was harvested 1, 3, or 7 days post LPS challenge and cells were analyzed by flow cytometry.

In some studies, the non-adherent cells from a day 8 GM-CSF BMDC cultures from GFP^+ BM cells were collected, resuspended in sterile PBS and 5 x 10^6 cells/ 50 μl PBS were instilled into the lungs of C57BL/6J mice. 24 h later, some animals were further challenged with 25 μg of LPS in 50 μl PBS via intratracheal instillation. The BAL and mediastinal lymph node were analyzed 24 h after LPS challenge and analyzed by flow cytometry.

To monitor changes in alveolar macrophages during LPS-induced lung inflammation, alveolar macrophages were isolated from several GFP^+ mice. Cells were spun down and treated with RBC lysis buffer (0.84% ammonium chloride) for 5 min at RT, counted and then resuspended in endotoxin free PBS. 3 x 10^5 GFP^+ alveolar macrophages in 50 μl PBS were

injected intratracheally into C57BL/6J mice. 24 h after the macrophages were instilled, some animals were further challenged with 25 μg of LPS in 50 μl PBS via intratracheal instillation. The BAL was collected at the indicated time points and analyzed by flow cytometry.

2.2.17 Competitive instillation of GM-CSF BMDCs into the lung

$CD11c^{+}$ $GR1^{-}$ MHC $II^{mid/low}$ cells were sorted from C57BL6/J ($CD44^{+/+}$, $CD45.2^{+}$) and $CD44^{-/-}$ ($CD45.2^{+}$) day 7 GM-CSF BMDC cultures by FACS. Equal numbers of C57BL/6J and $CD44^{-/-}$ BMDCs were combined and 3 x 10^5 total cells in 50 μl sterile PBS were instilled intratracheally into the lungs of BoyJ mice, and the BAL was collected 24 h or 1 wk later, analyzed by flow cytometry.

2.2.18 Instillation of peritoneal macrophages into the lung

Peritoneal macrophages were isolated from GFP^{+} mice by peritoneal lavage, cells were spun down and treated with RBC lysis buffer (0.84% ammonium chloride), $F4/80^{+}$ macrophages were purified as described in 2.2.3 and then resuspended in endotoxin free PBS. Each BoyJ mouse received 8 x 10^5 $F4/80^{+}$ GFP^{+} peritoneal macrophages in 50 μl PBS into the lung by intratracheal instillation. The BAL was collected 2 wk later, and analyzed by flow cytometry.

2.2.19 Competitive instillation of alveolar macrophages into the lung

Alveolar macrophages from multiple C57BL/6J and $CD44^{-/-}$ mice were harvested from the BAL and pooled. Cells were spun down and treated with RBC lysis buffer (0.84% ammonium chloride), counted and then resuspended in endotoxin free PBS. Equal numbers of C57BL/6J and

$CD44^{-/-}$ alveolar macrophages were combined and 3 x 10^5 total cells in 50 µl PBS were instilled into the lungs of BoyJ mice. The BAL was collected 1 wk later, and analyzed by flow cytometry.

2.2.20 Competitive bone marrow reconstitution

BoyJ mice received two doses of 650 rads of gamma irradiation. BM were harvested from C57BL/6J and $CD44^{-/-}$ mice, and after lysing the RBCs with 0.84% ammonium chloride for 5 min at RT, equal numbers of C57BL/6J and $CD44^{-/-}$ BM cells were mixed together and 8 x 10^6 cells resuspended in sterile PBS were injected intravenously into lethally irradiated BoyJ mice. The BAL, lung tissue and BM were collected 7 weeks post reconstitution and analyzed by flow cytometry.

2.2.21 Statistics

Data are shown as the average +/- standard deviation (SD) or standard error of the mean (SEM). Significance was determined by a student's *t*-test with $*p < 0.05$, $**p < 0.01$, $***p < 0.001$. Unless otherwise specified, significance was calculated by a two-tail, unpaired *t*-test.

Chapter 3: Hyaluronan binding and uptake by lipopolysaccharide and interferon gamma-induced inflammatory and interleukin 4-mediated alternatively activated macrophages

3.1 Introduction and rationale

Macrophages are a heterogeneous population with diverse morphology and functionality, and this is largely because of their ability to respond to various endogenous and exogenous signals. Macrophages have the ability to promote or dampen inflammation, which makes them an attractive therapeutic target. Some signals such as LPS and IFNγ, TNFα and GM-CSF can promote inflammatory macrophage function, while IL-4, prostaglandin E2 and apoptotic neutrophils can stimulate their differentiation into healing or anti-inflammatory macrophages (Mosser and Edwards, 2008). Specifically, the M1and M2 macrophage *in vitro* system characterizes macrophages polarized with IFNγ and LPS as classically activated M1 macrophages that perform inflammatory functions, and those stimulated with the Th2 cytokines IL-4 and/or IL-13, as alternatively activated M2 macrophages, also known as healing macrophages (Gordon, 2003; Gordon and Martinez, 2010). This *in vitro* model is a useful to investigate other potential functions and regulators of inflammatory and healing macrophages.

During an inflammatory response, HA synthesis is increased and HA can be broken down by hyaluronidase or ROS, which results in the accumulation of HA and HA fragments at the site of inflammation. CD44 and HA have been implicated in pro-inflammatory macrophage functions, including the recruitment of macrophages to inflammatory sites (Cuff et al., 2001; Jameson et al., 2005) and LMW HA induces the production of inflammatory chemokines and cytokines (Jiang et al., 2007). CD44 may also have a role in the resolution of inflammation by macrophages, such as the removal of apoptotic neutrophils (Hart et al., 1997) and the removal of excess HA and HA

fragments (Culty et al., 1992). Alveolar macrophages can internalize and degrade HA to regulate local HA concentration/ deposition in the lung. CD44 has been shown to be necessary to remove excess HA that accumulate in the lung during bleomycin-induced lung inflammation. Moreover, $CD44^{-/-}$ mice experience unresolved inflammation leading to death, with HA accumulating in the lungs. Interestingly, this phenotype can be reversed upon BM reconstitution with $CD44^{+/+}$ hematopoietic cells (Teder et al., 2002). These results support the idea that CD44 plays a role in the uptake of HA by healing macrophages during the resolution of inflammation, however there is no direct evidence demonstrating a role of macrophages in the removal of inflammatory LMW HA. To better understand the role of CD44 and HA in pro- and anti-inflammatory macrophage function, I will determine if M1 and M2 macrophages differentially regulate their ability to bind and uptake HA and HA fragments, and explore the mechanism by which macrophages bind HA.

3.2 Results

3.2.1 M1 and M2-polarizing agents induce CD44-mediated hyaluronan binding to different extents in mouse bone marrow-derived macrophages

To begin to understand the role of CD44 and HA binding in macrophage function, first I wanted to determine if CD44 plays a role in the differentiation of macrophages. $CD44^{+/+}$ and $CD44^{-/-}$ BM cells were cultured in the presence of M-CSF for five days to differentiate into macrophages, and then further stimulated in the presence of LPS plus IFNγ or IL-4 to be polarized into M1 and M2 macrophages, respectively. Although some phenotypic changes were observed after 24 h, these were further enhanced at 48 h and so this time point was chosen for further study. The expression of various known macrophage markers were examined on unstimulated (-), M1 and M2 BMDMs by flow cytometry and the data is summarized in Table 3.1. Compared to

unstimulated BMDMs, M1 BMDMs upregulated the expression of the common macrophage markers F4/80 and CD11b (Figure 3.1A) as well as the myeloid cell differentiation marker Gr1, which is highly expressed on monocytes and neutrophils (Figure 3.1A and B). In contrast, M1 polarizing agents downregulated the expression of the M-CSF receptor, CD115 on macrophages, as LPS causes the shedding of CD115 (Rovida et al., 2001) (Figure 3.1B). Gr1 was not highly expressed in macrophages and given that different macrophage subsets have slightly different levels of background staining, the change in Gr1 expression was better demonstrated when the expression of Gr1 was plotted against unlabeled controls of the corresponding macrophage subtype (Figure 3.1B). M1 BMDMs increased their Gr1 expression compared to unstimulated and M2 BMDMs. Unlike M1 BMDMs, M2 BMDMs showed elevated levels of CD11c, a common DC marker, while maintaining similar expression of F4/80 and CD115 as unstimulated BMDMs (Figure 3.1A). LPS plus IFNγ also induced the expression of CD14 (Figure 3.1B), a protein that facilitates the recognition of LPS as well as the activation markers CD40, CD80 and CD86 in M1 BMDMs. Very little or no expression of these activation markers were detected on unstimulated or M2 BMDMs (Figure 3.1). MHC II was upregulated in both M1 and M2 BMDMs, but IL-4 often induced slightly higher MHC II expression compared to LPS and IFNγ. Overall, polarized M1 and M2 BMDMs have a distinct phenotypic profile. Most notably, LPS plus IFNγ-stimulated M1 BMDMs were F4/80^{high} CD115^{low} CD11c^{low} CD14^{+} CD40^{high} CD80^{+} CD86^{+} MHC II$^{low/mid}$ whereas IL-4-activated M2 BMDMs were F4/80^{low} CD115^{high} CD11c^{high} CD14^{-} CD40^{low} CD80^{-} CD86^{-} MHC II^{mid} (Table 3.1 and Figure 3.1). CD44$^{-/-}$ BMDMs expressed the same surface markers as CD44$^{+/+}$ BMDMs upon differentiation into M1 and M2 macrophages (data not shown), suggesting CD44 was not necessary for the differentiation and activation of macrophages.

	- (Unstim) BMDM	M1 (LPS+IFNγ) BMDM	M2 (IL-4) BMDM
Macrophage Surface Markers			
F4/80	+	++	+
CD115	++	+	++
CD11b	++	+++	++
CD11c	- /+	-/+	+++
Gr1	+/+	++	+
CD14	-	+	-
Activation Markers			
CD40	+	+++	+
CD80	+	++	- /+
CD86	-/+	++	-/+
MHC II	-	+/+	++
CD44 and Hyaluronan binding			
CD44	+	++	+++
HA binding	-/+	+++	++

Table 3.1 Phenotype of unstimulated, M1 and M2 BMDMs
The expression of various surface markers in BMDMs derived from C57BL/6J mice stimulated with 100 ng/ml LPS plus 50 ng/ml IFNγ or with 10 ng/ml IL-4 for 2 days were analyzed by flow cytometry. This is a summary of one representative experiment repeated at least three times. +, ++ and +++ represents the relative positive expression between the different macrophage subsets, with + being the lowest and +++ highest expression. – indicates little or no detectable expression. -/+ is an intermediate phenotype between – and +, +/+ is an intermediate phenotype between + and ++.

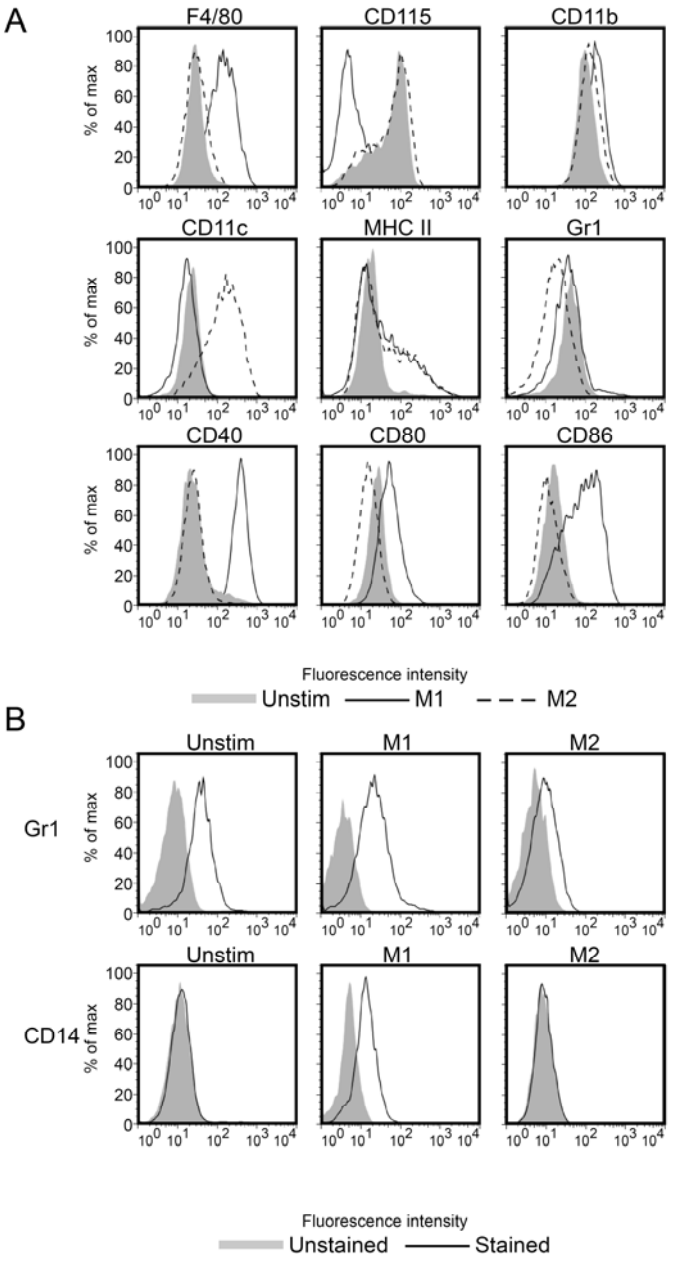

Figure 3.1 Phenotype of unstimulated, M1 and M2 BMDMs
BMDMs derived from C57BL/6J mice stimulated with 100 ng/ml LPS and 50 ng/ml IFNγ (M1 BMDM), or 10 ng/ml IL-4 (M2 BMDM) for 2 days. **A**, The expression of various surface markers compared between unstimulated (shaded), M1 (solid line) and M2 (dotted line) BMDMs by flow cytometry. **B**, Unstimulated, M1 and M2 BMDMs labeled with Gr1 and CD14 mAbs (solid line) were compared to unstained control (shaded) from the corresponding sub type of macrophages. This is a representative experiment repeated at least three times.

CD44 expression and its ability to bind Fl-HA were examined in M1 and M2 BMDMs 24 h and 48 h post stimulation, as well as in unstimulated BMDMs. Unstimulated macrophages expressed the HA receptor CD44, but did not have high binding for HA (Table 3.1 and Figure 3.2). After 48 h of culture with LPS and IFNγ, M1 BMDMs upregulated the expression of CD44 and exhibited significant levels of HA binding. Stimulation by IL-4 also enhanced CD44 expression in M2 BMDMs (often more so than the M1 BMDMs), and although these cells were induced to bind HA, they did not bind HA to the same extent as M1 BMDMs (Figure 3.2). HA binding by BMDMs was CD44 dependent, as $CD44^{-/-}$ M1 or M2 BMDMs did not bind HA (Figure 3.2).

3.2.2 CD44 reduces TNFα production but does not affect the production of nitric oxide or arginase activity by bone marrow-derived macrophages

To examine if CD44 regulated the function of macrophages, 24 h following stimulation, $CD44^{+/+}$ and $CD44^{-/-}$ M1 and M2 BMDMs were assessed for arginase activity, and the cell supernatants were collected to measure the production of NO and TNFα. As expected, M2 macrophages demonstrated higher arginase activity compared to M1 BMDMs, as indicated by the higher amount of urea produced from L-arginine (Figure 3.3A). The lack of CD44 did not affect the arginase activity of M2 BMDMs (Figure 3.3A). M1 but not M2 BMDMs produced pro-inflammatory mediators NO and TNFα (Figure 3.3B and C). Although the production of NO was similar between $CD44^{+/+}$ and $CD44^{-/-}$ M1 BMDMs (Figure 3.3B), slightly less TNFα was produced by $CD44^{-/-}$ M1 BMDMs (Figure 3.3C). Relative to $CD44^{+/+}$ M1 BMDMs, $CD44^{-/-}$ macrophages secreted 85% as much TNFα (Figure 3.3D). In the absence of CD44, M1 BMDMs still produced a significant amount of TNFα, and the biological significance of the 15%

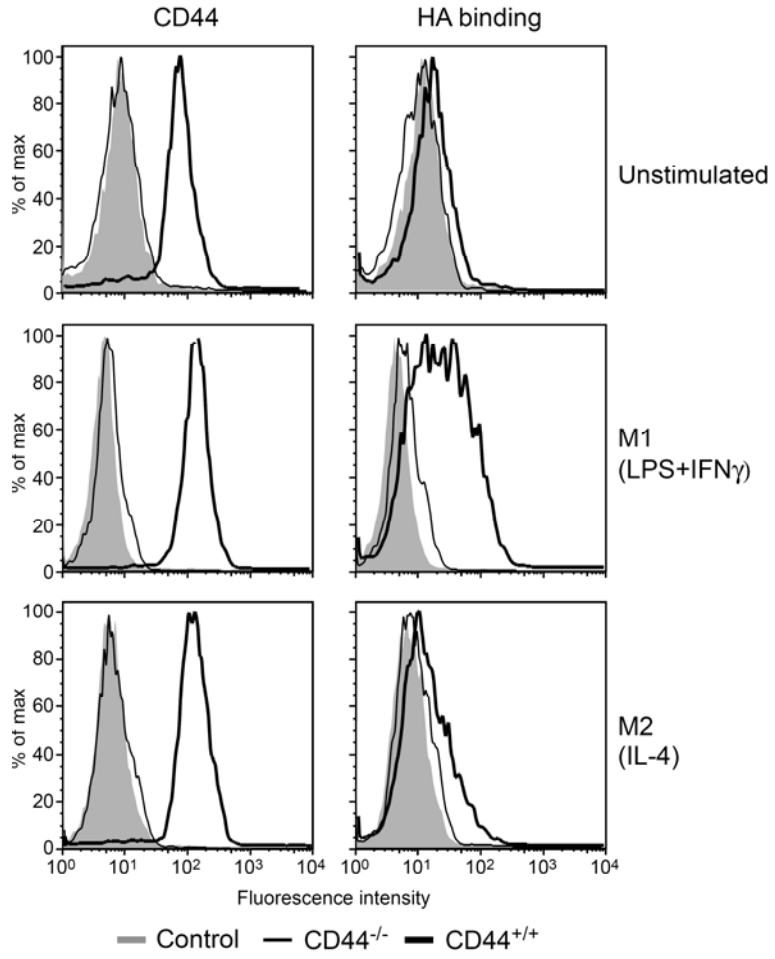

Figure 3.2 Induction of HA binding and CD44 expression in BMDMs
CD44 expression and HA binding were analyzed by flow cytometry in BMDMs derived from C57BL/6J ($CD44^{+/+}$) or $CD44^{-/-}$ mice stimulated with 100 ng/ml LPS and 50 ng/ml IFNγ, or 10 ng/ml IL-4 for 2 days. The left panel shows CD44 expression levels and the right panel shows Fl-HA binding on unstimulated, LPS plus IFNγ stimulated M1 and IL-4 stimulated M2 BMDMs from $CD44^{+/+}$ (thick line) and $CD44^{-/-}$ mice (thin line). Negative controls were unlabeled cells (shaded). This is one representative experiment of at least three.

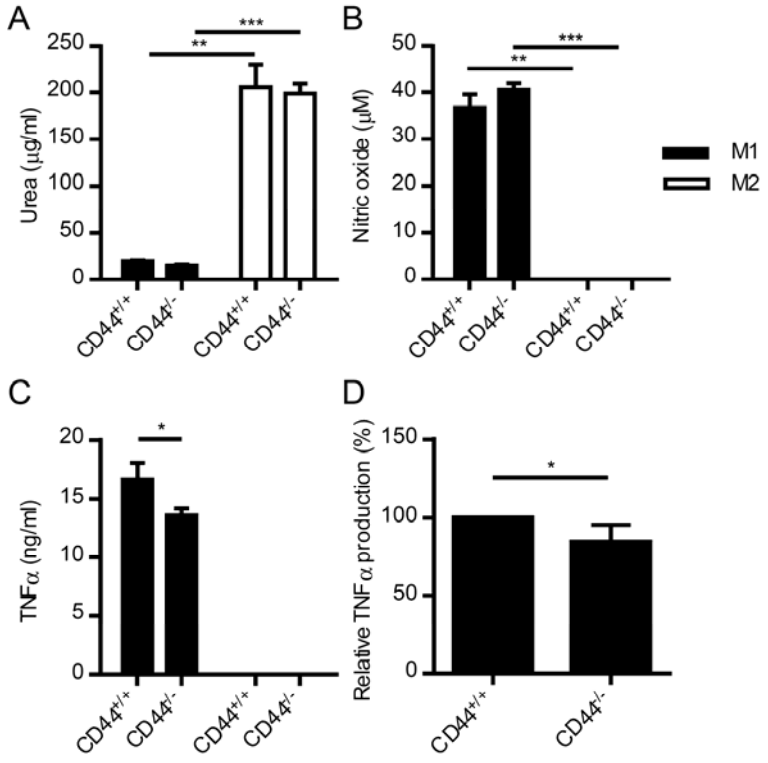

Figure 3.3 TNFα, NO production and arginase activity in M1 and M2 BMDMs
C57BL/6J ($CD44^{+/+}$) and $CD44^{-/-}$ BMDMs were stimulated with either 100 ng/ml LPS and 50 ng/ml IFNγ (M1) or 10 ng/ml IL-4 (M2) for 24 h. **A**, Cell lysates were analyzed for arginase activity. **B,C,** The amounts NO **(B)** and TNFα **(C)** in the tissue culture supernatants were analyzed by NO assay and ELISA, respectively. **D**, Relative TNFα production by $CD44^{-/-}$ M1 BMDMs over multiple experiments, with $CD44^{+/+}$ M1 BMDMs TNFα normalized to 100%. **A-C** is representative data from one mouse assayed in triplicates. The experiment was repeated at least four times. **D** is an average of five mice from five independent experiments. Data is shown as the mean +/- SD with significance indicated as * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

reduction would require further investigation. These results indicate that CD44 does not have a strong influence on TNFα and NO production by inflammatory M1 macrophages or arginase activity in M2 BMDMs.

3.2.3 The glycosaminoglycan inhibitor, β-D-xyloside, increases hyaluronan binding by bone marrow-derived macrophages

Because the most dramatic difference between $CD44^{+/+}$ and $CD44^{-/-}$ BMDMs was their ability to bind HA, I wanted to further investigate the mechanism and function of HA binding in M1 and M2 macrophages. To determine if glycosaminoglycan modification on CD44 was regulating HA binding in M1 and M2 BMDMs, macrophages were polarized in the presence or absence of 2 mM β-D-xyloside, a competitive inhibitor of glycosaminoglycan addition that prevents the addition of CS to CD44. After 48 h treatment, the presence of xyloside did not affect CD44 expression on macrophages but enhanced the HA binding ability of unstimulated and M2 BMDMs without affecting M1 BMDMs (Figure 3.4A). This suggested that the ability of CD44 to bind HA was negatively regulated by glycosaminoglycans in unstimulated and M2 macrophages. Interestingly, HA binding in LPS and IFNγ stimulated BMDMs were not affected by xyloside (Figure 3.4A), which led to the hypothesis that M1 BMDMs may reduce glycosaminoglycan addition to CD44 to induce the binding of HA.

Since xyloside does not specifically block the addition of CS to CD44, it was necessary to immunoprecipitate CD44 and examine its CS modification, to determine if CS modified CD44 was reduced on M1 BMDMs and increased on M2 BMDMs. The addition of CS to CD44 was detected using anti-CS mAb 3B3 (after chondroitinase ABC treatment to reveal the reactive CS stub) and analyzed by Western blot (Figure 3.4B). The CS expression from similar amounts of

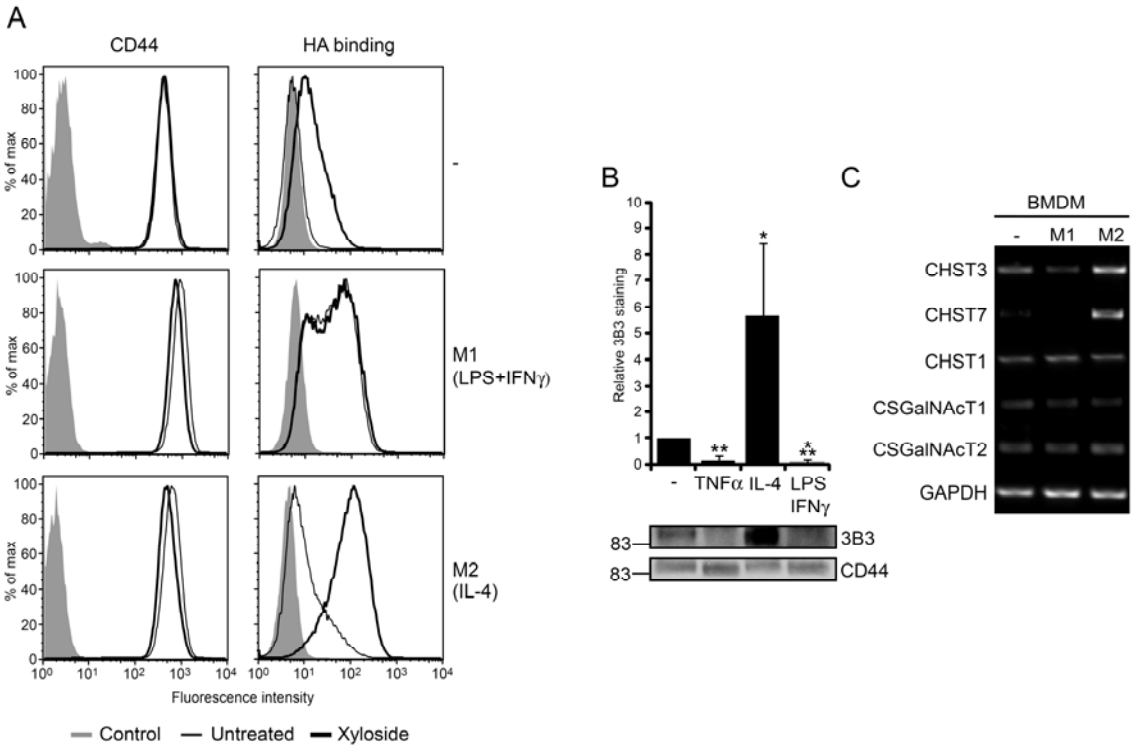

Figure 3.4 Differential CS sulfation in CD44 regulates HA binding in M1 and M2 BMDMs
C57BL/6J BMDMs were stimulated with either 100 ng/ml LPS and 50 ng/ml IFNγ (M1), 10 ng/ml IL-4 (M2) or 20 ng/ml TNFα. **A**, CD44 expression and HA binding were analyzed by flow cytometry in unstimulated (-), M1 and M2 BMDMs stimulated for 2 days in the presence (thick line) or absence (thin line) of 2 mM β-D-xyloside. The left panel shows CD44 expression, while the right panel shows binding to Fl-HA. Unlabeled cells were used as negative controls (shaded). This is one representative experiment repeated three times. **B**, Analysis of CS addition to CD44 from BMDMs stimulated with TNFα, IL-4, or LPS and IFNγ. Western blots were analyzed by densitometry and, after taking into account variations in CD44 loading, the intensity of 3B3 staining for CD44 from unstimulated BMDMs was set to 1. Data is shown as the mean +/- SD of three experiments with significance indicated as *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. **C**, *CHST3, CHST7, CHST1, CSGalNAcT1* and *CSGalNAcT2* mRNA expression from unstimulated (-), M1 and M2 BMDMs. Isolated mRNA was subjected to semiquantitative PCR and *GAPDH* expression was used as a loading control. This is representative data that was reproduced with BMDMs from four mice.

CD44 from untreated BMDMs was normalized and compared to macrophages polarized under different conditions. Consistent with previously published data where TNFα reduced CS addition to CD44 on monocytes (Delcommenne et al., 2002), BMDMs stimulated with 20 ng/ml TNFα also exhibited reduced CS (Figure 3.4B). Similarly, a loss of CS staining was observed in LPS and IFNγ polarized M1 BMDMs (Figure 3.4B). In contrast, IL-4 increased the expression of CS by approximately 5 fold (Figure 3.4B). In summary, CS modification on macrophages was downregulated by inflammatory stimulus such as TNFα and LPS plus IFNγ, but increased in the presence of IL-4, a cytokine used to differentiate macrophages into healing macrophages.

The 3B3 antibody recognizes the exposed 6-sulfated galactose/ N-acetylgalactosamine (GalNAc) in the CS stub, raising the possibility that changes to the sulfation of this sugar may also affect the degree of chondroitin sulfation. To better understand the mechanism by which LPS plus IFNγ and IL-4 could regulate the chondroitin sulfation of CD44, we measured the relative expression of two sulfotransferases, chondroitin 6-sulfotransferase 3 *(CHST3)* and 7 *(CHST7)*, both capable of sulfating GalNAc (Kusche-Gullberg and Kjellen, 2003); two enzymes that mediate the synthesis and elongation of CS, GalNAc transferase 1 *(GalNAcT1)* and GalNAc transferase 2 *(GalNacT2)* (Kusche-Gullberg and Kjellen, 2003); and *CHST1*, a Gal 6-sulfotransferase for keratan sulfate that is not detected by 3B3 mAb. *CHST3* and *CHST7* were both present at low levels in unstimulated BMDMs and while they were upregulated in M2 BMDMs, the expression of these sulfotransferases were downregulated in M1 BMDMs (Figure 3.4C), similar to the trend observed with the 3B3 CS antibody (Figure 3.4B). On the other hand, the expression of *GalNAcT1*, *GalNacT2* and *C6ST1* was constant for the unstimulated, LPS plus IFNγ and IL-4 stimulated BMDMs, suggesting that the change in sulfation detected by 3B3 in these macrophages was a result of CS sulfation and not due to changes in the elongation of CS,

or to the sulfation of keratan sulfate (Figure 3.4B). Together, these results demonstrated that CS on CD44 negatively regulates HA binding and that M1 and M2 BMDMs differentially regulate CS sulfation on CD44 to modify its ability to bind HA. LPS and IFNγ stimulated the downregulation of CS sulfation by reducing the expression of CS sulfotransferases *CHST3* and *CHST7* and this presumably promoted the ability of M1 BMDMs to bind HA. By contrast, IL-4 upregulated *CHST3* and *CHST7*, which led to an increase in CS sulfation and reduced HA binding, demonstrated by showing that HA binding was significantly enhanced when CS addition was inhibited by xyloside. In conclusion, HA binding by M1 and M2 macrophages is dynamically regulated by the extent of chondroitin sulfation on CD44.

3.2.4 LPS substantially enhances hyaluronan binding by M2 macrophages but IL-4 has little effect on M1 macrophages

M1 and M2 BMDMs are not terminally differentiated. M2 BMDMs, which normally do not produce inflammatory cytokines can produce TNFα upon subsequent exposure to LPS, demonstrating the plasticity of macrophages (Brown et al., 2011). We have found that LPS plus IFNγ and IL-4 stimulate different CS modifications on CD44 to regulate HA binding. Therefore, we wanted to determine if subsequent exposure to LPS or IL-4 affects M1 and M2 BMDM HA binding. Earlier in this study, I identified F4/80 as a surface marker that was increased in M1 BMDMs whereas CD11c expression was enhanced on M2 BMDMs (Table 3.1 and Figure 3.1). The addition of IL-4 to M1 BMDMs did not change the expression of F4/80 or CD11c on the macrophages. However, LPS increased F4/80 expression and downregulated CD11c expression on M2 BMDMs (Figure 3.5A). When macrophages were differentiated in the presence of both LPS and IL-4, they acquired a phenotype more similar to M1 BMDMs, suggesting LPS has a

more dominant effect on BMDMs compared to IL-4 (Figure 3.5A). This was also reflected in HA binding and CD44 expression, where LPS-treated M2 BMDMs had a dramatically enhanced ability to bind HA as well as elevated CD44 expression (Figure 3.5B and C). To evaluate if this increased HA binding was due to downregulation of CD44 chondroitin sulfation by LPS, M2 BMDMs treated with LPS were cultured in the presence or absence of xyloside. As expected, xyloside did not further enhance HA binding in LPS-treated M2 BMDMs, suggesting that LPS stimulation removed the inhibitory signals induced by IL-4 (Figure 3.5D). Xyloside increased HA binding in IL-4 activated M2 BMDMs (Figure 3.4A), but it did not affect the HA binding in IL-4 treated M1 BMDMs, which means IL-4 was not able to further induce CS modifications on CD44 in M1 BMDMs (Figure 3.5D). These results illustrate an additive effect of LPS and IL-4 on HA binding by macrophages, and this effect was most dramatic when BMDMs were primed with IL-4 and then activated with LPS.

3.2.5 CD44 mediates the trafficking of hyaluronan to the lysosomes

M1 and M2 macrophages bound HA to different extents and this process was dependent on differential modifications of CD44. To determine if these different forms of CD44 influenced the uptake of HA by M1 and M2 macrophages, cells were cultured with Fl- HA for 24 h, surface HA was then removed with hyaluronidase and the amount of intracellular HA analyzed by flow cytometry (Figure 3.6A). The M1 BMDMs took up more HA than the unstimulated or M2 BMDMs. In three experiments, relative to M1 macrophages, M2 macrophages took up 35% less HA (Figure 3.6B). Surprisingly, CD44 was not required for the uptake of HA, as $CD44^{-/-}$ M1 and M2 BMDMs both took up the same amount of HA as $CD44^{+/+}$ BMDMs (Figure 3.6A). Further analysis by confocal microscopy showed that intracellular HA was peri-nuclear in $CD44^{+/+}$ M1

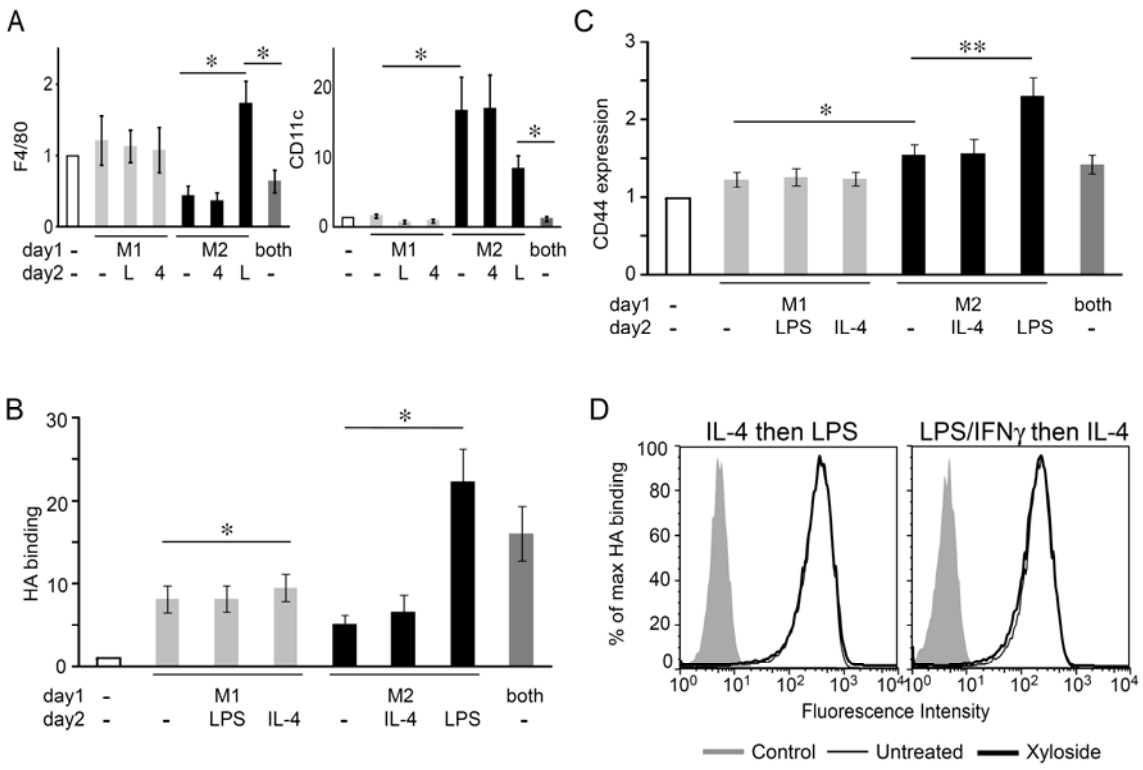

Figure 3.5 Distinct phenotypes of M1 and M2 BMDMs and relative expression of CD44 and HA binding with additional LPS or IL-4 stimulation

A-C, BMDMs were stimulated with either 100 ng/ml LPS and 50 ng/ml IFNγ (M1), 10 ng/ml IL-4 (M2), 100 ng/ml LPS, 50 ng/ml IFNγ and 10 ng/ml IL-4 (both) or media control (-) on day 1, then re-stimulated with LPS (L), IL-4 (4) or media alone (-) on day 2. **A**, Relative increase in expression levels of F4/80 (left panel) and CD11c (right panel) of activated versus unstimulated BMDMs. **B**, **C**, Relative increase in Fl-HA binding **(B)** and CD44 expression levels **(C)** compared to unstimulated BMDMs. **A**, was an average of three mice from three experiments, **B,C** was an average of six mice from six experiments. Graphs show the mean fluorescent intensity +/- SEM with significance indicated as * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. **D**, BMDMs were stimulated in the presence (black line) or absence (thin line) of 2 mM β-D-xyloside and the negative control (shaded) was unlabeled cells. Cells were stimulated with IL-4 followed by LPS on the second day (IL-4 then LPS) or stimulated with LPS and IFNγ followed by IL-4 on the second day (LPS/ IFNγ then IL-4). This is one representative plot of four experiments.

BMDMs, but in $CD44^{-/-}$ M1 BMDMs the HA was scattered within the cell. This suggests a role for CD44 in the intracellular trafficking of HA (Figure 3.6C). In $CD44^{+/+}$ but not $CD44^{-/-}$ BMDMs, intracellular HA co-localized with lysosomal-associated membrane protein 1 (LAMP-1), a lysosome marker (Figure 3.6C). Dextran is taken up into LAMP-1^{+} lysosomes (Zimmerli et al., 1996) and after 24 h of fluorescent dextran uptake, there was no difference in the localization of dextran in $CD44^{+/+}$ and $CD44^{-/-}$ M1 BMDMs, which implies that $CD44^{-/-}$ cells were not defective in transporting cargo to the lysosome (Figure 3.6D). Thus, M1 macrophages take up more HA than M2 macrophages independently of CD44, but CD44 was necessary for efficient intracellular trafficking of HA into lysosomes in M1 BMDMs.

3.2.6 M1 BMDMs take up more HMW HA whereas M2 BMDMs are more efficient at the uptake of LMW HA

Macrophages have different roles throughout an inflammatory response, this led to the hypothesis that inflammatory macrophages may interact with HMW HA to generate LMW fragments and promote inflammation, whereas non inflammatory macrophages play a role in the degradation of inflammatory LMW HA to restore HA homeostasis. Thus, the ability of $CD44^{+/+}$ and $CD44^{-/-}$ M1 and M2 BMDMs to take up HMW and LMW HA of defined sizes was examined. Macrophages were cultured with HMW (1.68×10^6 Da) or LMW (28.6 kDa) HA conjugated to Alexa Fluor 649 for 24 h, followed by hyaluronidase treatment to remove extracellular HA and the relative amount of internalized HA was measured by flow cytometry. M1 BMDMs took up more HMW HA compared to M2 BMDMs (Figure 3.7), consistent with the previous experiment using FL- HA (majority $>1 \times 10^6$ Da) (Figure 3.6). Interestingly, more LMW HA was taken up by M2 BMDMs than M1 BMDMs (Figure 3.7). Again, CD44 did not regulate the

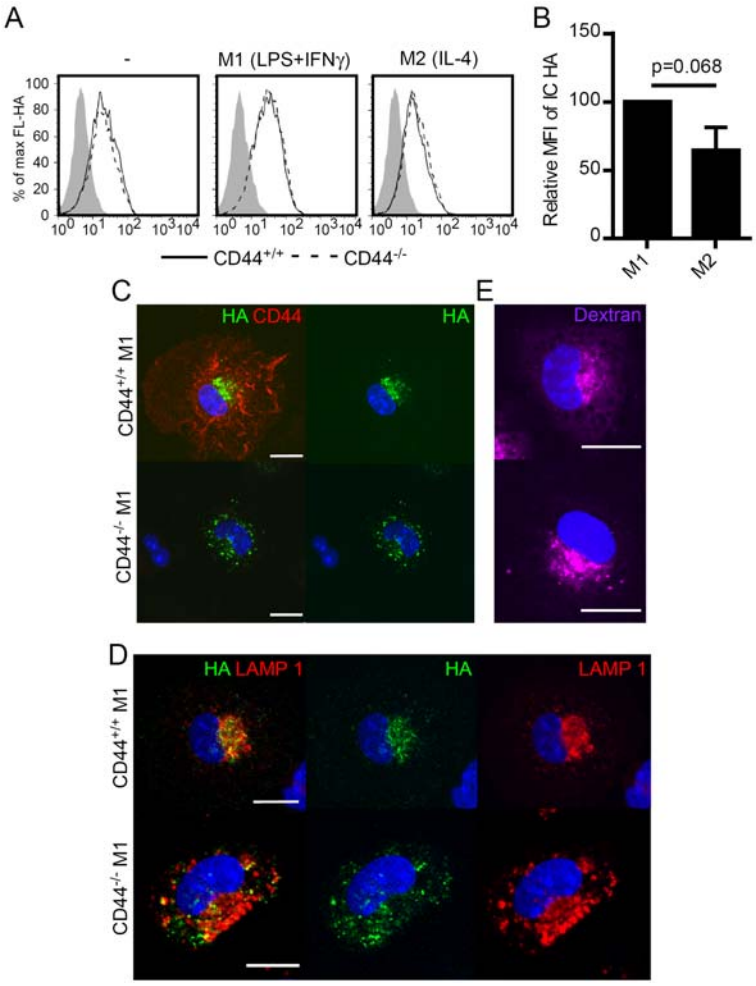

Figure 3.6 CD44 facilitates HA trafficking to the lysosomes
C57BL/6J ($CD44^{+/+}$) and $CD44^{-/-}$ BMDMs were stimulated with either 100 ng/ml LPS and 50 ng/ml IFNγ (M1) or 10 ng/ml IL-4 (M2) for 24 h, and were incubated with 5 μg/ml Fl-HA or Alexa Fluor 649 conjugated dextran at 37°C with 5% CO_2 for another 24 h. Cells were then treated with hyaluronidase for 5 min on ice to remove surface bound Fl-HA and then labeled with CD44 or LAMP-1 mAb. **A**, Intracellular HA in $CD44^{+/+}$ (solid) and $CD44^{-/-}$ (dotted) unstimulated, M1 and M2 BMDMs were analyzed by flow cytometry. Negative controls were unlabeled cells (shaded). **B**, Relative mean fluorescent intensity (MFI) of intracellular (IC) HA in $CD44^{+/+}$ M1 and M2 BMDMs. This is an average of three independent experiments, with the MFI of M1 BMDMs normalized to 100%. **C**, Confocal images of $CD44^{+/+}$ (top) and $CD44^{-/-}$ (bottom) M1 BMDM intracellular Fl-HA and CD44. **D**, Confocal images of $CD44^{+/+}$ (top) and $CD44^{-/-}$ (bottom) BMDM intracellular Fl-HA and LAMP-1. **E**, Confocal images of $CD44^{+/+}$ (top) and $CD44^{-/-}$ (bottom) M1 BMDM after 24 h dextran internalization. Scale bar represents 10 μm. This is one representative cells from one experiment, that was repeated two **(E)** or three times **(C and D)**, observed in at least 50 cells per experiment.

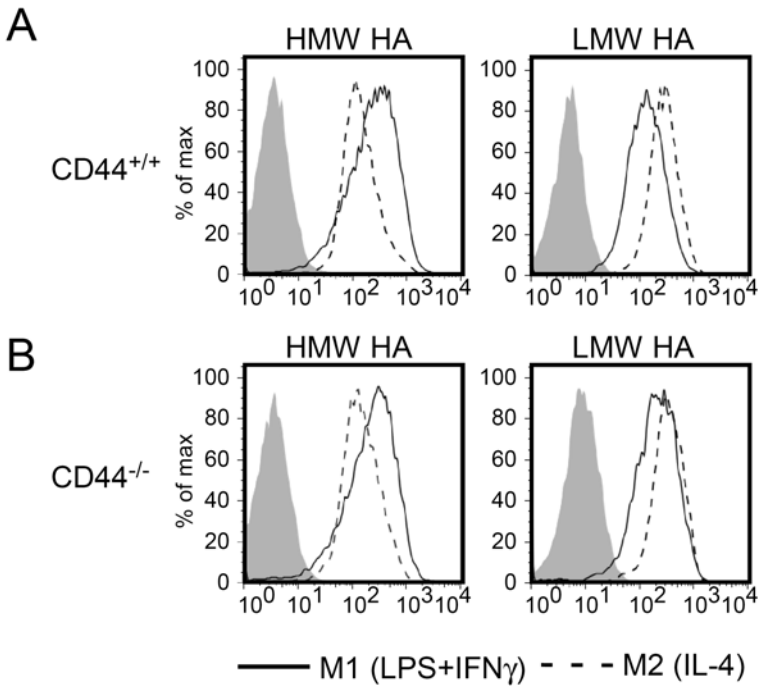

Figure 3.7 M1 BMDMs take up more HMW HA whereas M2 BMDMs are more efficient at the uptake of LMW HA

C57BL/6J ($CD44^{+/+}$) and $CD44^{-/-}$ BMDMs were stimulated with 100 ng/ml LPS and 50 ng/ml IFNγ (M1) or 10 ng/ml IL-4 (M2) for 24 h, then large (1.68 x 10^6 Da, HMW) or small (28.6 kDa, LMW) HA conjugated to Alexa Fluor 649 were added and further cultured at 37°C with 5% CO_2 for 24 h. The amount of large and small intracellular HA in M1 BMDMs (solid line) or M2 BMDMs (dotted line) was analyzed by flow cytometry after treating with *Streptomyces* hyaluronidase for 5 min on ice to remove surface bound HA. Negative control was unlabeled cells (shaded). This is one representative experiment, repeated at least three times.

uptake of HMW or LMW by M1 or M2 BMDMs as the trends were consistent between $CD44^{+/+}$ and $CD44^{-/-}$ BMDMs (Figure 3.7). Taken together, inflammatory M1 BMDMs took up more HMW HA and conversely, M2 BMDMs took up more LMW HA. Future experiments will address if M1 BMDMs indeed generate HA fragments upon the uptake of HMW HA, determine if M2 BMDMs degrade LMW HA more efficiently than M1 BMDMs, and assess the importance of CD44 in HA trafficking and turnover.

3.3 Discussion

These results demonstrate that LPS and IFNγ-stimulated inflammatory macrophages as well as IL-4 activated macrophages not only have differential ability to bind HA, but that they also differ in their ability to uptake HMW and LWM HA. Specifically, LPS plus IFNγ reduced CS sulfation on CD44 by downregulating the transcriptional expression of the chondroitin sulfotransferases *CHST3* and *CHST7* and this led to increased HA binding in M1 macrophages. In contrast, IL-4 enhanced CS sulfation on CD44 by increasing the mRNA expression of the same chondroitin sulfotransferases and this negatively correlated with HA binding. CS modification on CD44 negatively regulates macrophage HA binding and it is one mechanism by which macrophages and monocytes (Delcommenne et al., 2002) regulate HA binding. Similar to LPS plus IFNγ stimulated macrophages, TNFα also induced macrophages to bind HA and the increase in HA binding also correlated with reduced CS sulfation on CD44 (Figure 3.4B), suggesting that HA binding was regulated through a similar mechanism. LPS and IFNγ stimulate macrophages to produce TNFα (Figure 3.3C), therefore either LPS activates a similar pathway as TNFα, or perhaps TNFα, acting in an autocrine manner, is the major cytokine that regulates HA binding in inflammatory macrophages. TNFα has been shown to stimulate human peripheral blood monocytes to bind HA (Brown et al., 2001; Delcommenne et al., 2002; Levesque and Haynes, 1997), and while the addition of TNFα neutralizing antibody inhibited 92% of TNFα induced HA binding, the antibody was only able to inhibit 24% of LPS-induced HA binding (Levesque and Haynes, 1997), which means that there must be other factors other than TNFα regulating LPS-induced HA binding.

Interestingly, LPS treatment dramatically enhanced IL-4 induced HA binding to a higher level than LPS plus IFNγ stimulated macrophages. These macrophages upregulated both M1 and

M2 surface markers, demonstrating that the M2 macrophages differentiated *in vitro* retain some developmental plasticity. In this study, IL-4 induced the expression of CD11c, a classical DC marker and reduced the expression of F4/80, a common marker used to identify macrophages. There has been a lot of discussion as to what identifies a macrophage versus a DC, and this is partly due to the use of non-specific markers to define a cell type (Geissmann et al., 2010a). In support of this, M-CSF derived BMDMs that did not express CD11c became $CD11c^{+}$ after just 24 h in the presence of IL-4. This illustrates a potential limitation in the use of CD11c as a DC marker, as an increase in $CD11c^{+}$ population *in vivo* during an immune response may be due to an increase in IL-4 stimulated macrophages rather than DC. The addition of LPS to M2 BMDMs readily downregulated the expression of CD11c (Figure 3.5A) further illustrating that the expression of surface markers on macrophages are easily influenced by change in environmental signals.

Surprisingly, CD44 was not needed for either M1 or M2 macrophages to take up HA, and instead CD44 was more important for the intracellular trafficking of HA. Although the amount of intracellular HA was similar in $CD44^{+/+}$ and $CD44^{-/-}$ macrophages (Figure 3.6), if $CD44^{-/-}$ macrophages fail to transport HA to the appropriate cellular compartment for degradation, it would still lead to an accumulation of HA in the supernatant. Currently there is not enough evidence to conclude if CD44 is needed for the fragmentation or degradation of HA by M1 and M2 macrophages, and additional experiments that specifically measure the change in the amount and size of HA would be necessary to address this question. It was interesting that even though M2 macrophages did not bind Fl-HA (Figure 3.2, Fl-HA from rooster comb is mostly HMW) or HMW HA (data not shown) as well as M1 BMDMs, and neither M1 or M2 BMDMs bound to LMW HA (data not shown), M2 macrophages consistently internalized more LMW HA than M1

BMDMs (Figure 3.7). However, given that HA uptake was CD44 independent, this is perhaps not so surprising. The data support the hypothesis that M2 macrophages may be more efficient at the removal of HA fragments than M1 BMDMs. M1 BMDMs bound higher levels of HMW HA, which could potentially mediate M1 tissue retention at the inflammatory site and/or contribute to the generation of HA fragments.

It has been suggested that CD44 is required to bind HMW HA and bring HA closer to Hyal-2 to cleave and produce HA fragments (Stern, 2004). Hyal-2 is located on the cell membrane and generates 20 kDa HA fragments. In contrast, Hyal-1degrades HA into tetrasaccharide, and because the optimal pH for Hyal-1 is at 3.7, it is suggested to be in acidic compartments such as the lysosomes (Stern et al., 2007). CD44 has been shown to associate with hyaluronidases and help mediate HA fragmentation in a kidney cell line, as Hyal-1 and Hyal-2 were not able to degrade HA without the co-transfection of CD44 (Harada and Takahashi, 2007). If CD44 mediated HA binding functions to generate HA fragments, then it is surprising that the higher HA binding M1 macrophages were trafficking HA into the lysosomes, where Hyal-1 degrades HA into tetrasaccharides (Figure 3.6). Perhaps, CD44 plays a role in both the fragmentation and trafficking of HA in M1 BMDMs. M1 macrophages might first generate 20 kDa HA fragments by CD44 and Hyal-2 at the cell surface, then CD44 subsequently traffics the intermediate HA (20kDa) into lysosomes for degradation. CD44-deficient M1 macrophages might not be able to generate 20kDa HA fragments, and because the size of HMW-HA is too big or due to the lack of CD44 to facilitate intracellular trafficking, the HA does not enter into the lysosome for degradation.

In this study, the plasticity and heterogeneity of macrophages was observed on multiple levels. In particular, CD44 mediated HA binding and HA uptake by macrophages were

differentially regulated by inflammatory stimuli LPS plus IFNγ and IL-4. These results demonstrated the ability of macrophages to dynamically interact with HA in response to different environmental signals, and suggest that CD44, through its interaction with HA, may be important for different macrophage functions during various stages of an immune response.

Chapter 4: Hyaluronan binding in subpopulations of GM-CSF derived dendritic cells: hyaluronan binding identifies a macrophage-like proliferative population and hyaluronan degradation prevents proliferation and promotes maturation of $CD11c^+$ cells

4.1 Introduction and rationale

Many macrophages and DCs reside in the tissue during homeostasis. Upon an inflammatory stimulus, DCs in particular are known to effectively migrate to the draining lymph nodes to initiate the adaptive immune response. Macrophages and DCs rely on signals from their surroundings to stimulate the appropriate response. In Chapter 3, I demonstrated that M-CSF BMDMs differentially modify CD44 CS sulfation in response to macrophage polarizing agents LPS plus IFNγ and IL-4, and this allows macrophages to regulate their ability to bind soluble HA. GM-CSF along with cytokines IL-1α, IL-1β, IL-3 and TNFα, have been shown to induce monocyte HA binding (Levesque and Haynes, 1997, 1999). GM-CSF is known as a hematopoietic growth factor that is important for the development and activation of myeloid cells like macrophages and DCs and is categorized as a pro-inflammatory cytokine (Burgess and Metcalf, 1980; Hamilton, 2008; Inaba et al., 1992; Lutz et al., 1999).

HA is a major component of the ECM found in almost all tissues. During homeostasis, HA exists as a 1 x 10^6 Da HMW polymer and it is dynamically regulated at the site of inflammation and tissue injury where HA fragments of different sizes are generated (Jiang et al., 2007; Taylor and Gallo, 2006). These HA fragments are also known as DAMPs that induce sterile inflammation (Chen and Nunez, 2010). The addition of LMW HA of 4-14 oligosaccharides to GM-CSF BMDC cultures stimulated the maturation of DCs to induce the production of inflammatory cytokines IL-1β, TNFα and IL-12, and upregulated the expression

of co-stimulatory molecules CD40, CD80 and CD86 in a TLR-4 dependent manner (Termeer et al., 2002; Termeer et al., 2000). In another study, DCs differentiated in the presence of GM-CSF express genes involved in the synthesis and degradation of HA, and the addition of Pep-1 a positively charged peptide that blocks hyaluronan interaction inhibited T cell activation by reducing T cell proliferation, IL-2 and IFNγ production in a DC co-culture. (Mummert et al., 2002). Furthermore, the blockage of HA interaction with Pep-1, limited the egress of Langerhans cells out of the tissue in a skin inflammation model (Mummert et al., 2000). Thus, GM-CSF stimulates monocyte HA binding, LMW HA activates GM-CSF derived DCs, DCs express HA synthesis and degradation genes, interaction with HA may regulate Langerhans cell trafficking and *in vivo* DCs localized in the tissue where HA is abundant. These results support a GM-CSF induced DC-HA interaction, but currently very little is known about the ability or function of DCs to bind HA, or the role of DCs in HA turnover. In this chapter, I investigated the HA binding ability in GM-CSF derived immature and mature dendritic cells and examined the function of endogenous HA as well as HA binding in these cells.

4.2 Results

4.2.1 GM-CSF induces hyaluronan binding in bone marrow-derived dendritic cell cultures and hyaluronan binding identifies a sub-population of CD11c$^+$, MHC II$^{mid/low}$ cells

Early in a GM-CSF BMDC culture (day 0-5), the majority of the non-adherent cells were positive for Gr1, which marks neutrophils and monocytes. The proportion of Gr1$^+$ cells in the culture was reduced as CD11c$^+$ cells were enriched over 12 days (Figure 4.1A). Neutrophils are short lived, and this can lead to a gradual decline in Gr1$^+$ cells. In addition, Gr1$^+$ monocytes in the BM are CD11b$^+$ and Ly6C^{high} and have been shown to further mature into CD11c$^+$ MHC II$^+$

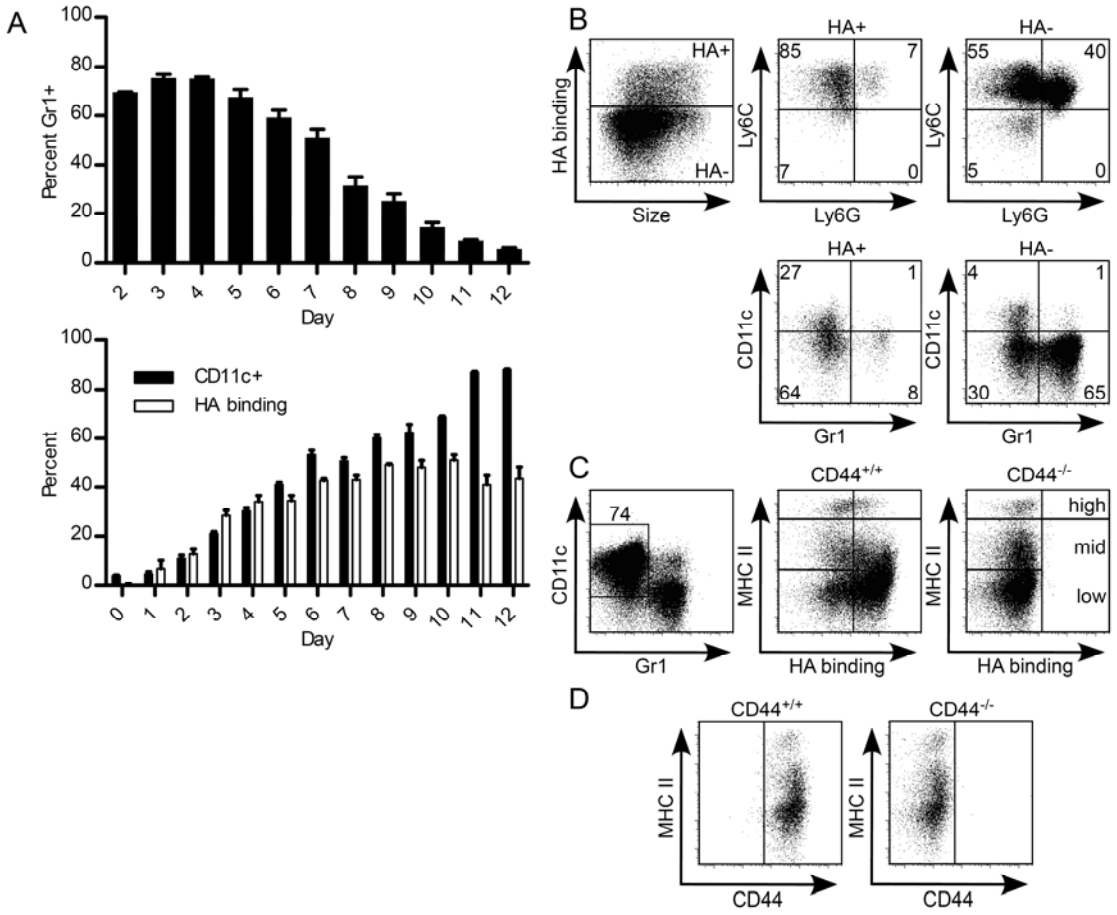

Figure 4.1 GM-CSF induces HA binding in BMDCs and HA binding identifies a sub-population of CD11c^{+}, MHC II$^{mid/low}$ cells

C57BL/6J (CD44$^{+/+}$) and CD44$^{-/-}$ BM cells were cultured in the presence of GM-CSF for 12 days. The phenotype and HA binding ability of these cells were analyzed by flow cytometry on different days. **A**, Percentage of GR1^{+} (top), CD11c^{+} and Fl-HA binding (bottom) cells over time. Graphs show the average +/- SD of three mice from one experiment. **B**, Day 4 HA binding (HA+) and non HA binding (HA-) cells were gated, and were analyzed for their Ly6C, Ly6G (top) and CD11c, Gr1 (bottom) expression. **C**, Day 7 CD11c^{+} Gr1^{-} cells were gated and the expression of MHC II and HA binding on the gated cells were analyzed. **D**, MHC II and CD44 expression in day 7 cells. This is representative data that was reproduced with six mice over two experiments, three mice were examined in each experiment.

DCs in the presence of GM-CSF and/or IL-4 (Hamilton and Achuthan, 2013; Xu et al., 2007). Therefore, the decline in $Gr1^+$ population and increase in $CD11c^+$ DCs (Figure 4.1A) is likely due to the death of neutrophils as well as the differentiation and expansion of $CD11c^+$ cells from monocytes. Similar to the $CD11c^+$ population, the Fl-HA binding population also increased over time (Figure 4.1A bottom). However, starting from day 5, there was always a higher proportion of $CD11c^+$ cells than HA binding cells. Therefore, HA binding was induced in GM-CSF BMDC cultures, but not all $CD11c^+$ cells were HA binding. To identify the cells that were HA binding, I first analyzed the day 4 culture and found 85% of the HA binding cells to be $Ly6C^+$ $Ly6G^-$ monocytes and non HA binding cells were a mix of $Ly6C^+$ $Ly6G^-$ monocytes and $Ly6C^+$ $Ly6G^+$ neutrophils (Figure 4.1B). Furthermore, the HA binding cells were mostly $Gr1^-$ with higher expression of CD11c in comparison to the non HA binding population, which was mostly $Gr1^+$ with little CD11c expression (Figure 4.1B). On day 4, HA binding marks monocytes that have low Gr1 and higher CD11c expression, suggesting HA binding is induced early in the differentiation of monocytes to $CD11c^+$ DCs.

In the GM-CSF BMDC cultures, both immature and mature DCs are present in the culture at day 7, and one of the markers used to distinguish these populations is MHC II, where mature DC exhibit higher MHC II expression than the immature DCs (Lutz et al., 1999). On day 7, 74% of the cells were $CD11c^+$ $Gr1^-$ DCs. Interestingly, only a sub population of these $CD11c^+$ $Gr1^-$ cells were HA binding and this was CD44 dependent (Figure 4.1C). The HA binding cells have relatively lower MHC II expression compared to the MHC II high population, suggesting they were less mature. Since immature DCs bind HA, this would explain the similar percentage in HA binding and total $CD11c^+$ cells at earlier time points, and as DCs mature and bind less HA, the percentage of CD11c becomes greater than the proportion of HA binding cells (Figure 4.1A).

Cells generated from CD44 deficient BM were able to differentiate into both MHC II^{high} mature and MHC $II^{mid/low}$ immature DCs similar to the $CD44^{+/+}$ BM cells, but $CD44^{-/-}$ cells were not able to bind HA (Figure 4.1C). The immature, MHC $II^{mid/low}$ DCs were able to bind more HA, while the more mature MHC II^{mid} and MHC II^{high} DCs bound less HA (Figure 4.1C). The differential ability to bind HA was not due to expression of the major HA receptor CD44 because all of the $CD11c^{+}$ $Gr1^{-}$ DCs expressed similar levels of CD44 (Figure 4.1D), so their ability to bind HA must be regulated through posttranslational modifications of CD44 (Johnson et al., 2000; Ponta et al., 2003; Pure and Cuff, 2001). These results suggest an induction of HA binding on monocytes maturing in the presence of GM-CSF, and that the MHC $II^{mid/low}$ $CD11c^{+}$ immature DCs but not the MHC II^{high} mature DCs have the ability to bind high levels of HA.

4.2.2 Hyaluronan binding MHC $II^{mid/low}$ cells have a phenotype characteristic of immature dendritic cells with less migratory ability

To better characterize the HA binding population, the expression of an activation marker CD86; common macrophage markers F4/80 and CD115; chemokine receptor CCR5 expressed on immature DCs, and CCR7 induced on mature DCs were analyzed by flow cytometry (Figure 4.2A). Consistent with a mature DC phenotype, the MHC II^{high} population expressed higher levels of the co-stimulatory molecule, CD86, and CCR7, which are necessary for mature DCs to migrate toward the chemokines CCL19 and CCL21 in the draining lymph nodes and to activate local T cells. The MHC $II^{mid/low}$ $CD11c^{+}$ cells did not express CD86 or CCR7, and were positive for CCR5. Mature DCs did not express F4/80 or CD115, where as the majority of the MHC $II^{mid/low}$ cells were positive for these markers. Within the MHC $II^{mid/low}$ population, some of the MHC II^{mid} cells expressed an intermediate phenotype. These MHC II^{mid} cells expressed lower

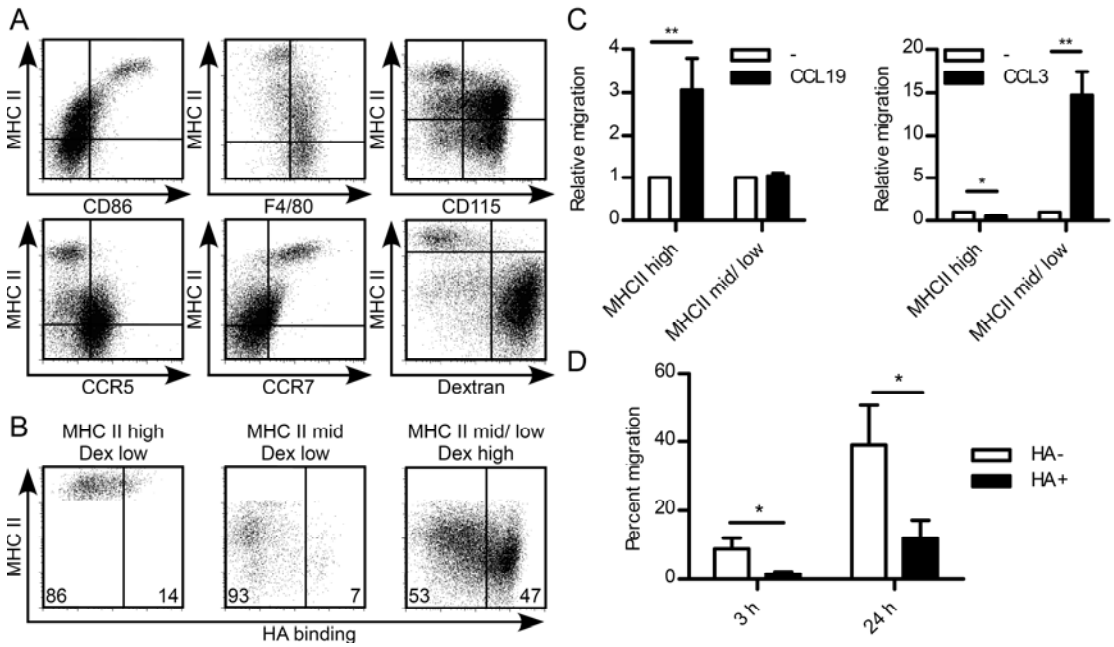

Figure 4.2 HA binding MHC II$^{mid/\ low}$ cells have a phenotype characteristic of immature DCs with less migratory ability

C57BL/6J BM cells were cultured in the presence of GM-CSF for 7-9 days. **A**, The expression of MHC II versus CD86 and CD115 were analyzed on day 7 CD11c$^+$ cells. Expression of MHC II versus CCR5 and CCR7 were analyzed on cells from day 7 cultures. MHC II and F4/80 expression were analyzed on day 8 CD11c$^+$ cells. Day 9 cells were incubated with Alexa Fluor 649 conjugated dextran at 37^{o}C for 45 min, the expression of MHC II and amount of dextran uptake were analyzed on CD11c$^+$ cells. **B**, HA binding ability of mature and immature DCs gated by MHC II expression and dextran in **A**. **C**, The migration of day 8 cells towards 40 ng/ml CCL19 after 2 h and 40 ng/ml CCL3 after 3 h were examined in a transwell assay. Number of CD11c$^+$ Gr1$^-$ MHC II^{high} and MHC II$^{mid/low}$ cells that migrated in the absence of chemokine was set to 1 to measure the specific migration towards the chemokine. **D**, Percentage of CD11c$^+$ Gr1$^-$ MHC II$^{mid/low}$ HA binding (HA+) and MHC II$^{mid/low}$ low HA binding (HA-) cells that migrated towards 40 ng/ml CCL3 after 3 h and 24 h out of the total number of HA+ and HA- cells plated. Data shown is from one representative experiment, repeated at least three times. Graphs in **C** and **D** show an average of three mice from one experiment ± SD with significance indicated as * $p < 0.05$, ** $p < 0.01$.

levels for F4/80, CD115 and CCR5 similar to a mature DC, but were not positive for CD86 or CCR7 (Figure 4.2A). MHC II^{mid} $CD11c^{+}$ cells were also mostly low HA binding cells (Figure 4.1C and data not shown).

Functionally, one way to distinguish immature DCs from mature DCs is by their ability to sample substances from the environment by macropinocytosis, such as the uptake of dextran. Immature DCs take up more dextran than mature DCs (Kato et al., 2000). Following 45 min incubation with fluorescent dextran at 37 degrees, there were three major populations separated by their MHC II expression and relative amount of dextran uptake (Dex), MHC II^{high} Dex^{low}, MHC II^{mid} Dex^{low} and MHC $II^{mid/low}$ Dex^{high} (Figure 4.2A). Again, a MHC II^{mid} population was more mature compared to the MHC $II^{mid/low}$ $CD11c^{+}$ cells as they did not take up dextran. The ability of these three populations to bind HA was examined to confirm the ability of mature and immature cells to bind HA (Figure 4.2B). All of the HA binding cells were within the MHC $II^{mid/low}$ Dex^{high} population, and the more mature Dex^{low} populations were mostly low HA binding, suggesting only immature cells were able to bind HA. However, not all of the MHC $II^{mid/low}$ Dex^{high} immature cells were high HA binding. Within the MHC $II^{mid/low}$ Dex^{high} population, the majority of the low HA binding cells were higher for MHC II expression compared to the high HA binding cells, correlating HA binding with lower MHC II expression.

As expected, in transwell migration assays, the MHC II^{high} mature DCs migrated towards the CCR7 ligand CCL19, while MHC $II^{mid/low}$ immature DCs migrated towards the CCR5 ligand, CCL3 (Figure 4.2C). Within the MHC $II^{mid/low}$ $CCR5^{+}$ cells that migrated, a greater percentage of low HA binding cells migrated after 3 h and 24 h compared to the high HA binding MHC $II^{mid/low}$ DCs (Figure 4.2D). Taken together, HA binding identifies an immature MHC $II^{mid/low}$ $CD11c^{+}$ DC population that does not express DC maturation markers, takes up dextran, and was

the least migratory towards CCL3. Furthermore, higher levels of MHC II correlated with lower HA binding affinity. This led to the hypothesis that HA identifies an immature DC population, and DCs down regulate HA binding upon maturation.

4.2.3 Hyaluronan binding identifies the most proliferative population that does not mature into MHC II^{high} cells

Features of the HA binding population were consistent with the function of an immature DC. To test the hypothesis that DCs down regulate their ability to bind HA as they mature, day 7 CD11c^{+} Gr1^{-} cells were sorted into three populations based on their MHC II expression and HA binding and then changes in HA binding were analyzed over time. CD11c^{+} Gr1^{-} MHC II$^{mid/low}$ high HA binding (p1), MHC II^{mid} low HA binding (p2) and MHC II^{high} low HA binding (p3) BMDCs were sorted by FACS. The same number of cells from each population were then plated in triplicate wells in the presence of GM-CSF, one well from each population was analyzed on day 8, 10 and 12. Supporting our hypothesis, three days after sorting (day 10) the HA binding cells (p1) downregulated HA binding as they increased their MHC II expression (Figure 4.3A). The MHC II^{mid} low HA binding population (p2) rapidly matured into MHC II^{high} after 24 h, and up to 67% of cells were MHC II^{high} by day 10, consistent with the expected maturation of DCs from MHC II^{mid} to MHC II^{high} (Figure 4.3A). The majority of the MHC II^{high} non/ low HA binding (p3) cells remained MHC II^{high} (Figure 4.3A) To our surprise, p1 increased their MHC II expression and reduced HA binding, but they never matured into MHC II^{high} cells even after 5 days (Day 12) (Figure 4.3A).

Since each well started with equal numbers on day 7, cell numbers within each population were comparable over time. Interestingly, relative to the number of p1 cells present in

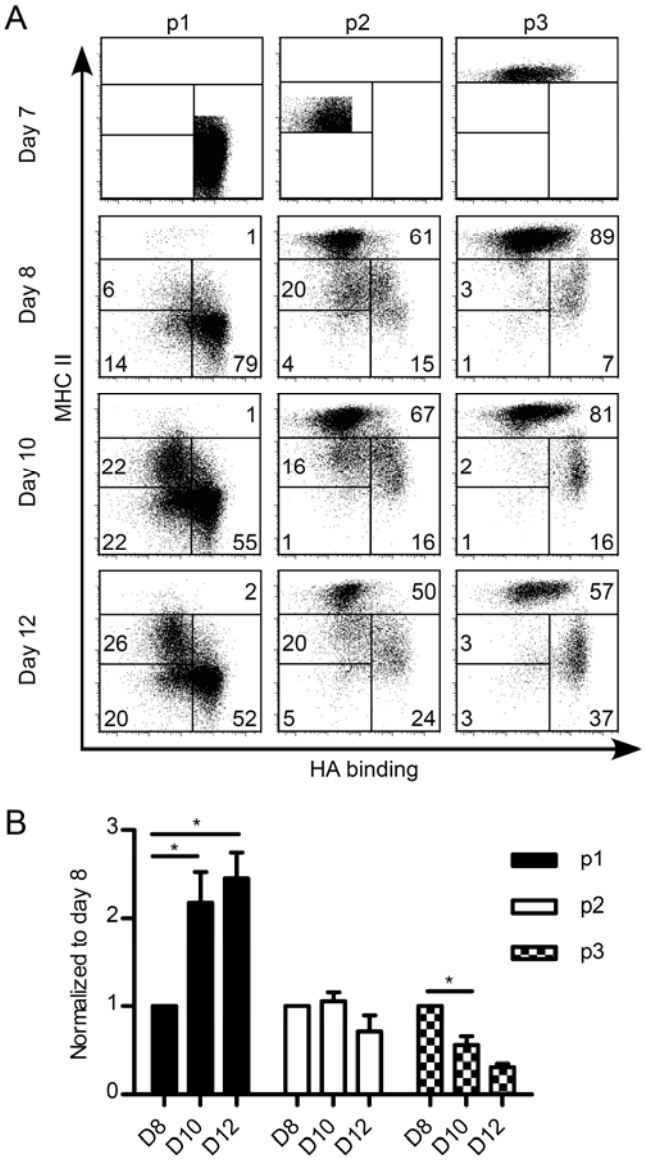

Figure 4.3 HA binding identifies a highly proliferative population that does not mature into MHC II^{high} cells

Day 7 C57BL/6J BMDCs were sorted for CD11c^{+} GR1^{-}, MHC II$^{mid/low}$ high HA binding (p1), MHC II^{mid} non/ low HA binding (p2) and MHC II^{high} non/ low HA binding (p3) populations. Approximately 5 x 10^{4} cells from each population were plated and further cultured with fresh GM-CSF. **A**, MHC II expression and HA binding were analyzed 1 day (day 8), 3 days (day10) and 5 days (day 12) later by flow cytometry. **B**, Proliferation was measured by the fold change in cell number relative to the number of cells present on day 8. This is a representative plot repeated three times, one mouse per experiment. Graph shows an average of three mice from three experiments ± SD with significance indicated as * $p < 0.05$, paired t-test.

day 8, there was a significant increase in cell number after 2 days (day 10), which means these cells were proliferating (Figure 4.3B). In contrast, the low HA binding p2 and p3 did not proliferate. The number of p2 cells in day 10 remained similar to day 8, with fewer cells present by day 12 relative to day 8. This indicates that p2 cells were either not proliferating, or proliferating and dying at the same rate from day 8-10 and eventually more cells died by day 12 (Figure 4.3B). The most mature population, p3, was also the least viable, evident by the gradually decrease in cell number over time (Figure 4.3B). The increase in death of the MHC II^{high} cells (p3) and the fact these cells remain MHC II^{high} (Figure 4.3A) suggest p3 cells were short lived, terminally differentiated cells. Cell sorting by flow cytometry yielded 95-98% purity, and there were likely some non MHC II^{high} cells that were included in p3. Because the MHC II^{mid} HA binding cells were more viable than the MHC II^{high} cells, this may have led to the drop in percentage of MHC II^{high} cells in the p3 population on day 12 and the expansion of a HA binding population (Figure 4.3A). These results were interesting and unexpected, as MHC $II^{mid/low}$ immature DCs were believed to all mature into MHC II^{high} DCs. Here HA binding identifies a proliferative $CD11^{+}$ MHC $II^{mid/low}$ population (p1) that down regulates HA binding as they gain MHC II, but do not mature into MHC II^{high} cells.

4.2.4 The hyaluronan binding population also synthesizes hyaluronan and its removal affects both dendritic cell number and maturation

The MHC $II^{mid/low}$ population in GM-CSF BMDC cultures binds high levels of HA. To understand the importance of HA binding, the source of HA was investigated. Given that GM-CSF BMDCs express hyaluronan synthase genes, *Has-1, 2 and 3* (Mummert et al., 2002), I wanted to determine if DCs expressed endogenous HA on their cell surface and whether the

mature and immature cells expressed similar levels of HA. Day 7 cells were labeled with hyaluronan binding protein (HABP) to measure the relative amount of HA on the cell surface or given Fl-HA to compare their ability to bind HA. HA was detected on the surface of $CD11c^+$ $Gr1^-$ cells using HABP (Figure 4.4A). Specifically, $CD11c^+$ cells with high MHC II expression had less HA on the surface relative to the MHC $II^{mid/low}$ cells (Fig 4.4A). Using MHC II expression as a guide, cells that synthesized more HA also bound higher levels of HA (Figure 4.4A). To determine if the removal of endogenous HA affects the $CD11c^+$ cells, day 7 cells were treated with hyaluronidase (HAase) purified from *Streptomyces* for 24 h. Relative to the day 7 cell number, while untreated cells proliferated, the presence of HAase inhibited proliferation (Figure 4.4B). Secondly, HAase treatment enhanced the maturation of DCs. In a representative experiment, 15% of the on adherent cells were MHC II^{high} on day 7 and this increased to 19% after 24 h. When day 7 cells were treated with HAase for 24 h, the proportion of MHC II^{high} cells was increased to 43 % (Figure 4.4 C). To determine if this effect was dependent on HA binding via CD44, CD44 deficient BMDCs were also treated with HAase for 24 h. HAase treatment promoted the maturation of both $CD44^{+/+}$ and $CD44^{-/-}$ cells, which means this effect was independent of CD44 mediated HA binding (Figure 4.4D).

4.2.5 Hyaluronidase limits the proliferation of the hyaluronan binding population and promotes the maturation of non or low hyaluronan binding $CD11c^+$ cells

The removal of endogenous HA in the culture limited the proliferation and promoted the maturation of total $CD11c^+$ cells (Figure 4.4B and C). Since proliferation and maturation were

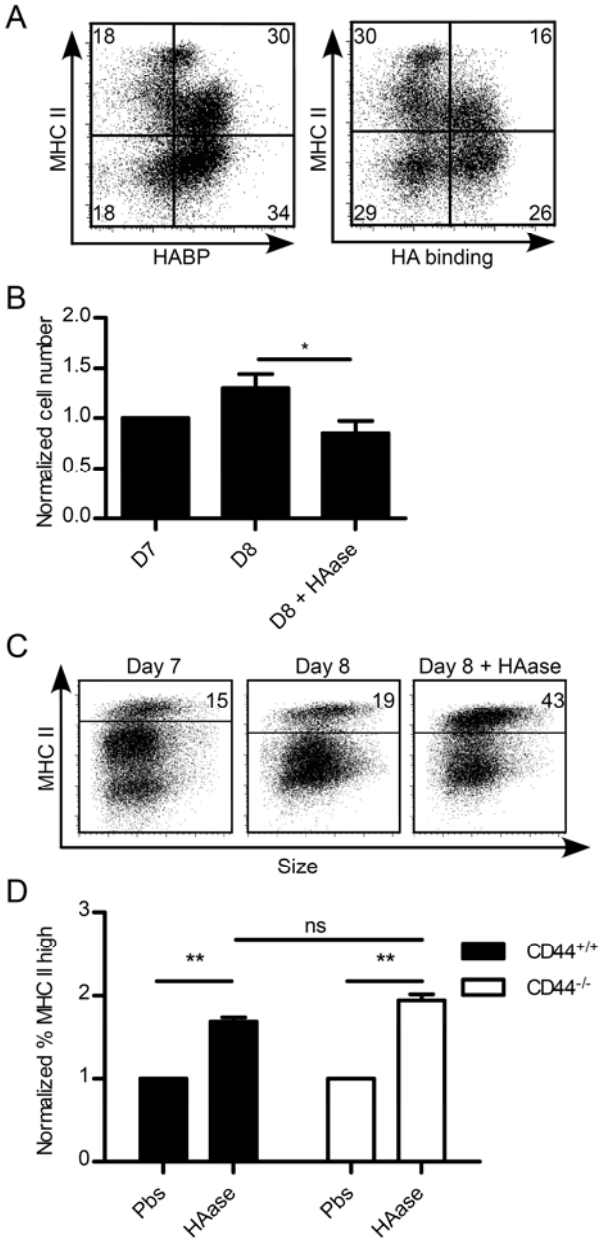

Figure 4.4 Removal of endogenous HA affects both DC proliferation and maturation
A, MHC II expression, HA binding and surface HA expression were analyzed on $CD11c^+$ $Gr1^-$ cells in a day 7 culture. Cells were labeled with Fl-HA to analyze HA binding and surface HA expression was detected by HA binding protein (HABP). **B-D**, C57BL/6J ($CD44^{+/+}$) or $CD44^{-/-}$ BMDCs from day 7 GM-CSF BMDC cultures were treated with hyaluronidase (HAase, *Streptomyces*, 20 U/ml) or PBS for 24 h. **B**, Change in $CD44^{+/+}$ BMDC cell number relative to day 7. **C**, The expression of MHC II in $CD11c^+$ $GR1^-$ C57BL/6J derived BMDCs. **D**, Relative increase in the percentage of $CD11c^+$ $GR1^-$ MHC II^{high} cells after 24 h HAase treatment, normalized to PBS treated samples in $CD44^{+/+}$ and $CD44^{-/-}$ BMDCs. **A,C** is one representative experiment, repeated at least three times. Graphs **B and D** show an average of three mice from one experiment ± SD, repeated two times with significance indicated as * $p < 0.05$, ** $p < 0.01$.

characteristic of the high and low HA binding populations (Figure 4.3), I wanted to determine if the removal of HA will have different effects on the HA binding and non or low HA binding populations. To address this question, day 7 cells were gated on the $CD11c^{+}$ $Gr1^{-}$, then four populations based on their MHC II expression and HA binding were isolated by FACS and treated with or without HAase daily until day 12 (Figure 4.5A). After three days in culture, the immature MHC II^{-} low HA binding cells (p0) differentiated into both MHC $II^{mid/low}$ HA binding (p1) as well as the MHC II^{high} low HA binding (p3) mature DCs. Similar to the previous experiments, the MHC $II^{mid/low}$ HA binding p1 upregulated MHC II expression and reduced HA binding ability but barely any cells became MHC II^{high} (Figure 4.5B). The fact that MHC II^{-} non/ low HA binding (p0) were able to give rise to p1, p2 and p3 suggests there was sufficient time for progenitor cells to mature into MHC II^{high} DCs but HA binding cells simply do not become MHC II^{high}.

LPS is a MAMP known to induce DC maturation, and perhaps the HA binding population requires a strong stimulus like LPS to mature. When sorted day 7 cells (Figure 4.6A) were stimulated with or without 100 ng/ml LPS for 24 h, LPS stimulated 11% more p0 cells and 30% more p2 cells to mature into MHC II^{high} cells compared to the untreated samples (Figure 4.6B). In contrast, there was only a 2% increase in percentage of MHC II^{high} cells the HA binding p1 population (Figure 4.6B). Compared to the day 8 cell number, LPS treatment decreased the viability of p0 and p2 by half, and limited the proliferation of the p1 HA binding population (Figure 4.6C). The absolute number of MHC II^{high} cells was obtained using the MACSQuant, and independent of the drop in total cell number in the presence of LPS, the absolute number of MHC II^{high} cells were still increased in p0 and p2 subsets (Figure 4.6D). Consistent with the literature, LPS is an inducer of DC maturation, however not all of the

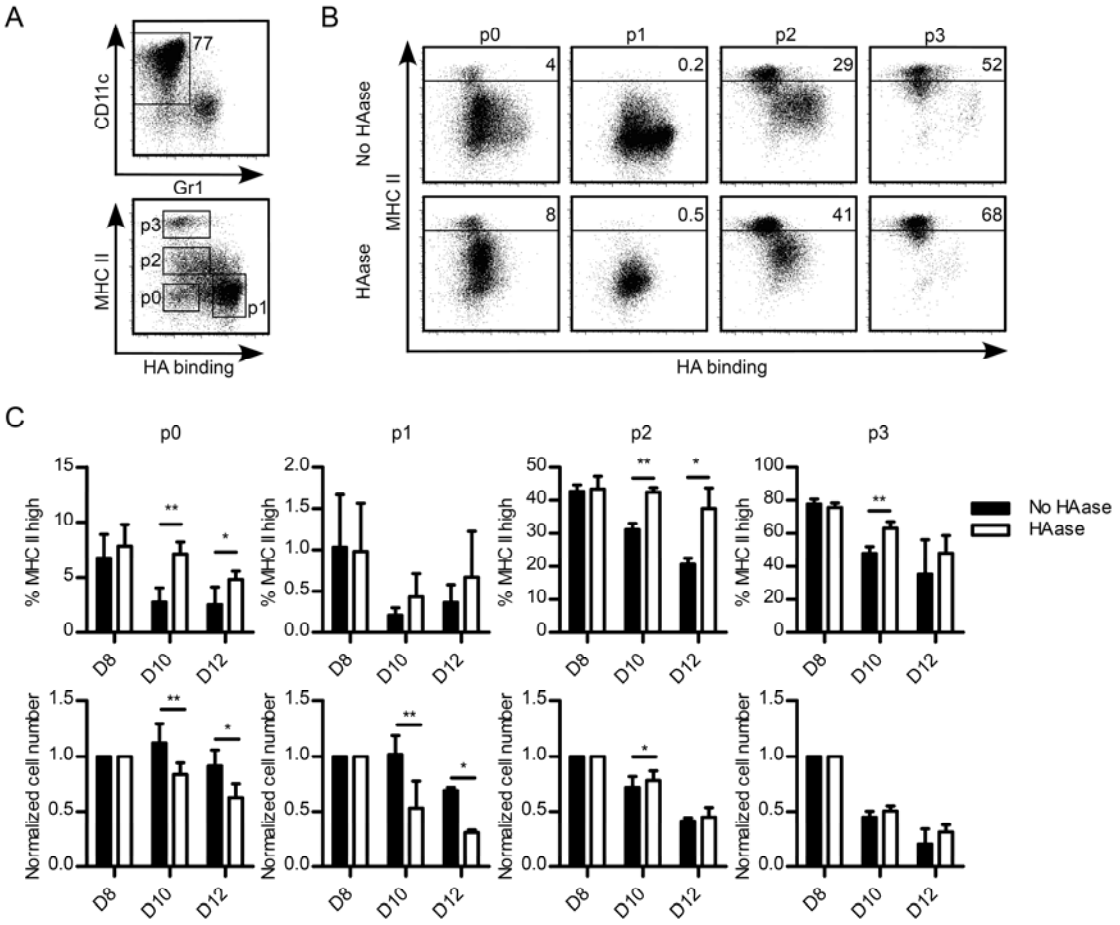

Figure 4.5 Hyaluronidase limits the proliferation of the HA binding population and promotes the maturation of non/ low HA binding CD11c$^+$ cells

A-C, Day 7 C57BL/6J BMDCs were sorted for CD11c$^+$ GR1$^-$, MHC II$^-$ non/ low HA binding (p0), MHC II$^{mid/low}$ HA binding (p1), MHC II^{mid} non/ low HA binding (p2) and MHC II^{high} non/ low HA binding (p3) populations (**A).** Equal cells from each population were plated and further cultured with fresh GM-CSF with or without daily hyaluronidase (HAase, Testicular, 20 U/ml) treatment. **B**, Day 10 (3 days post sort) BMDCs cultured in the absence (No HAase, top panel) or presence of HAase (bottom panel) were analyzed for MHC II expression and HA binding by flow cytometry. **C**, Percentage of MHC II^{high} cells on 1 day, (D8), 3 days (D10) and 5 days (D12) post sort (top panel). Number of viable cells were obtained from MACSQuant, and cell numbers were normalized to day 8. Graphs show an average of three mice from one experiment ± SD, with significance indicated as * $p < 0.05$, ** $p < 0.01$. This is one representative experiment repeated two times.

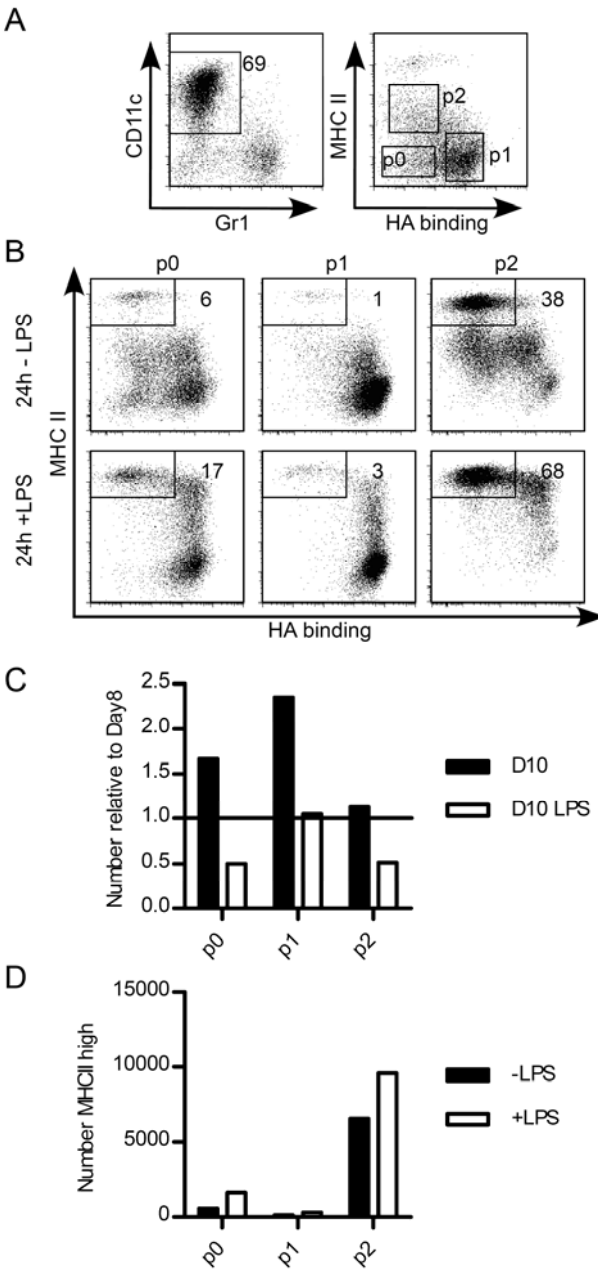

Figure 4.6 LPS promotes BMDC maturation and inhibits proliferation
A-C, Day 7 C57BL/6J BMDCs were sorted for $CD11c^{+}$ $GR1^{-}$, MHC II^{-} HA^{-} (p0), MHC $II^{mid/low}$ HA^{+} (p1), and MHC II^{mid} HA^{-} (p2) cells **(A)**. $5x10^{4}$ cells from each population were plated and further cultured with fresh GM-CSF, with or without LPS (100 ng/ml). **B**, Expression of MHC II and HA binding 24 h +/- LPS treatment analyzed by flow cytometry. **C and D**, The number of viable cells and MHC II^{high} cells cultured in the presence or absence of LPS after 3 days (D10) were analyzed using MACSQuant. **C**, Relative number of cells present on day 10, with day 8 numbers from each population set to 1. **D**, Absolute number of MHC II high cells in a day 10 cell culture. This is representative data from one experiment with one mouse, repeated twice.

MHC II mid and low expressing $CD11c^+$ cells that were classically known as the immature DCs were able to mature into MHC II^{high} DCs. Here we show that only the non/ low HA binding cells can efficiently mature into MHC II^{high} mature DCs.

The effect of HAase was similar to LPS stimulation; both HAase (Figure 4.5) and LPS treatment (Figure 4.6) promoted cell maturation and inhibited proliferation. To eliminate the possibility of endotoxin contamination in the HAase, endotoxin levels were measured by Limulus amebocyte lysate assay and measured less than 0.06 EU/ml for *Streptomyces* HAase and less than 1 EU/ml for bovine testicular HAase (data not shown). The HAase effect on unsorted BMDCs was consistently observed with HAase from *Streptomyces* (Figure 4.4) and HAase from bovine testes (data not shown). Because HAase from two very different sources exhibited similar effects on BMDCs, suggests an enzyme specific response rather than the contamination of both HAases. Finally, upon heat inactivation of HAase (boiled at 100°C for 30 min), the maturation effect was partially inhibited (data not shown), but its affect on BMDC proliferation remains to be established.

After culture in the presence of HAase for three days, a higher proportion of MHC II^{high} cells were present in the non HA binding populations, MHC II^- low HA binding p0, MHC II^{mid} low HA binding p2 and MHC II^{high} low HA binding p3 without effecting the MHC $II^{mid/low}$ high HA binding p1 (Figure 4.5 B). Quantitative analysis of the change in proportion of MHC II ^{high} cells and cell number relative to day 8 showed that HAase significantly promoted the maturation p0 and p2 cells into MHC II^{high} cells, and limited the proliferation/ survival of p0 and p1 cells (Figure 4.5C). The fact that more MHC II^{high} mature DCs differentiated from MHC II^- low HA binding (p0) cells than MHC $II^{mid/low}$ HA binding (p1) cells (Figure 4.5B), and that the addition of LPS (Figure 4.6B and D) as well as HAase (Figure 4.5C) only promoted the maturation of low

HA binding cells strongly suggest two lineages of $CD11c^+$ cells were present in the GM-CSF BM cultures. In addition to promoting the differentiation of DCs, GM-CSF is also commonly used to generate macrophages that are characterized as an adherent population that do not have a dendritic morphology. Looking at the appearance of the sorted populations, dendrites were apparent in the all of the MHC II^{high} cells (p3), but the HA binding p1 cells never developed any dendrites from day 7 to day 12 (data not shown). Earlier in the study I showed that HA binding MHC $II^{mid/low}$ cells expressed F4/80 and CD115 (Figure 4.2A), were active in macropinocytosis (Figure 4.2B), less migratory (Figure 4.2D). Here I have further shown that the MHC $II^{mid/low}$ HA binding cells do not mature into MHC II^{high} cells even upon LPS stimulation (Figure 4.6). Together, the HA binding cells have features similar to macrophages, therefore HA binding identifies a macrophage-like $CD11c^+$ population that is distinct from the classical DCs.

4.2.6 The alveolar space preferentially retains the hyaluronan binding cells and only the low hyaluronan binding MHC II^{high} cells migrate to the lymph node upon LPS-induced lung inflammation

HA is abundant in the lung tissues and has been shown to localize around blood vessels and alveolar spaces (Cheng et al., 2011). To gain insight into the function of CD44 mediated HA binding in GM-CSF derived $CD11c^+$ cells I decided to study its function in the lung where HA and GM-CSF are present. GFP^+ BM cells were cultured in the presence of GM-CSF for 8 days, then all of the cells in the culture, including $CD11c^-$ $Gr1^+$ neutrophils, high and low HA binding $CD11c^+$ cells were instilled into the lung to examine if the HA binding macrophage-like cells would be better retained in the lung (Figure 4.7). 99% of the cells differentiated from GFP BM

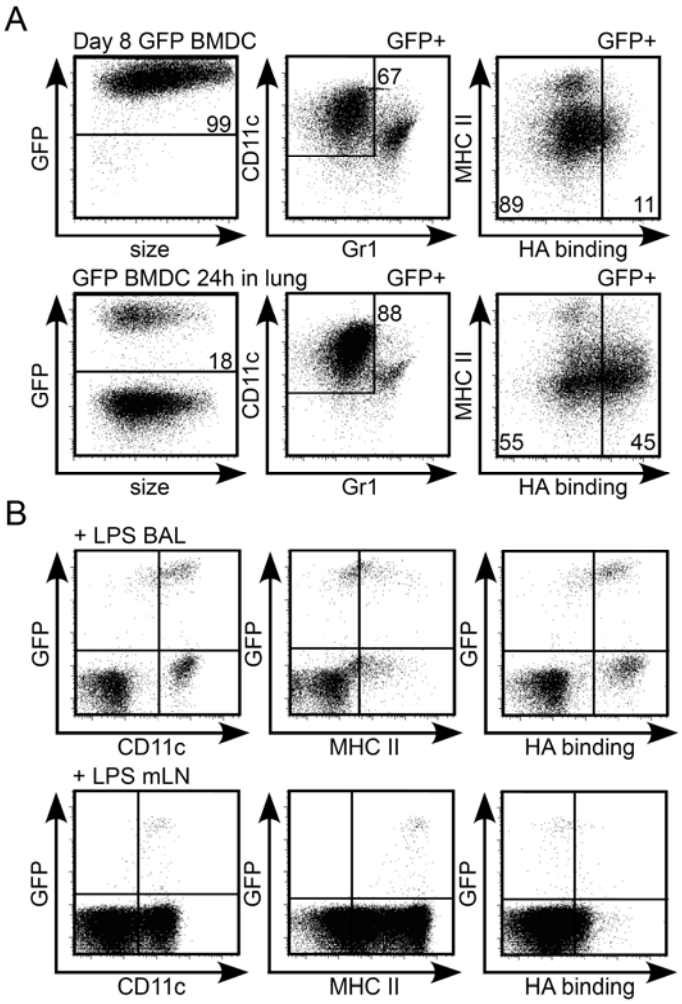

Figure 4.7 HA binding cells are retained in the alveolar space and only the non/ low HA binding MHC II^{high} cells migrated to the lymph node upon LPS-induced lung inflammation
A, 5 x 10^6 GFP$^+$ BM cells cultured in the presence of GM-CSF for 8 days were instilled into the lung. The proportion of CD11c$^+$ GR1$^-$ and HA binding cells in the GFP$^+$ population prior to instillation (top) and in the BAL after 24 h (bottom) were analyzed by flow cytometry. **B**, 24 h after the cells were instilled into the lung, LPS (25 μg/ 50 μl) was injected via intratracheal instillation. 24 h after LPS stimulation, the BAL (top) and mediastinal lymph node (LN, bottom) were analyzed for CD11c, MHC II expression and HA binding by flow cytometry. This is one representative experiment repeated three times.

cells expressed high levels of GFP, and on day 8 there were 67% $CD11c^+$ $Gr1^-$ cells with 11% of these cells binding HA (Figure 4.7A). 5 million of the GFP^+ cells were intratracheally instilled into the lung of GFP negative C57BL/6J mice. 24 h later, 18% of the cells collected from BAL were GFP^+, with 88% of the GFP^+ cells being $CD11c^+$ $Gr1^-$, and within the $CD11c^+$ cells, 45% were HA binding (Figure 4.7A). The proportion of $CD11c^+$ cells increased from 67% *in vitro* to 88% in the BAL suggesting $CD11c^+$ cells were preferentially retained in the alveolar space compared to the neutrophils. Similarly, the HA binding phenotype was selected for in the alveolar space as the percentage of HA binding cells increased from 11% to 45% (Figure 4.7A).

Upon inflammation, DCs are known to be more effective at migrating to draining lymph nodes than macrophages. GFP^+ cells were given 24 h to settle in the lung, then LPS was instilled into the lung to induce inflammation. 24 h following LPS stimulation the BAL and the mediastinal lymph node were analyzed. Indeed, the GFP^+ cells that remained in the alveolar space were $CD11c^+$ MHC $II^{mid/low}$ and HA binding (Figure 4.7 B). On the other hand, only $CD11c^+$ MHC II^{high} low HA binding cells migrated to the draining lymph node upon LPS-induced inflammation (Figure 4.7B). Interestingly, in contrast to the BAL where GFP^- $CD11c^+$ host cells were high HA binding, the GFP^- $CD11c^+$ cells in the lymph node were non or low HA binding. Thus the alveolar space selects for an HA binding phenotype, and these results were consistent with the hypothesis that HA binding cells identify a macrophage-like population that resides in the tissue.

4.2.7 CD44 positive CD11c^{+} MHC II$^{mid/low}$ cells were preferentially retained in the alveolar space over CD44 negative cells

Although HA binding cells were preferentially retained in the alveolar space, this does not directly show the importance of CD44 mediated HA binding in the retention of CD11c^{+} cells in the lung. To address this question, CD45.2^{+} CD44$^{+/+}$ and CD45.2^{+} CD44$^{-/-}$ CD11c^{+} MHC II$^{mid/low}$ cells were isolated by FACs and then equal number of CD44$^{+/+}$ and CD44$^{-/-}$ cells were competitively instilled into the lungs of BoyJ mice that lack the congenic marker CD45.2 for 24 h or 1 week. Prior to injection, there were 49% CD45.2^{+} CD44$^{+/+}$ cells and 51% CD45.2^{+} CD44$^{-/-}$ cells. 24 h later, within the CD45.2^{+} donor cell population, 64% of the cells were CD44$^{+/+}$ and 36% of the cells were CD44$^{-/-}$, demonstrating a CD44$^{+/+}$ cell advantage (Figure 4.8A). Not all CD44$^{+/+}$ MHC II$^{mid/low}$ cells bind HA, but cells that were selected for in the alveolar space were HA binding (Figure 4.8A). Consistent with the earlier observation (Figure 4.7), HA binding appears to be a favorable phenotype in the alveolar space (Figure 4.8A and Figure 4.7). After 1 week, the percentages of CD44$^{+/+}$ and CD44$^{-/-}$ cells were similar to the percentages after 24 h, (Figure 4.8A). Following the examination of multiple biological replicates, there were more CD44$^{+/+}$ cells than CD44$^{-/-}$ cells by percentage (Figure 4.8B) and number (Figure 4.8C) at both the 24 h and 1 week time points. The variation in cell number (Figure 4.8C) is likely due to technical inconsistencies during instillation and not because of CD44, because the CD44 advantage was consistently observed within the donor cell population by percentage (Figure 4.8B). This indicates a role for CD44 and possibly HA binding in facilitating the retention of these CD11c^{+} macrophage like cells in the alveolar space.

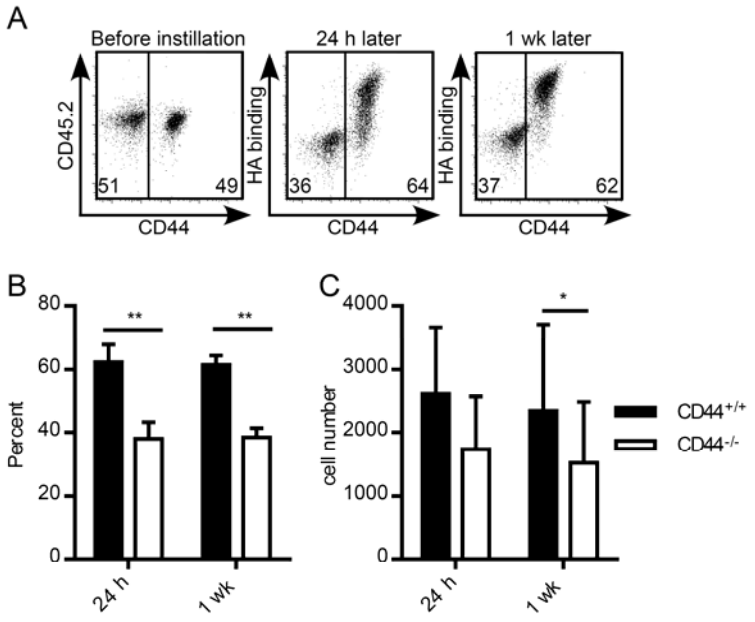

Figure 4.8 CD44 facilitates the retention of CD11c^{+} cells in the alveolar space
CD11c^{+} GR1^{-} MHC II$^{mid/low}$ BMDCs were sorted from day 7 CD45.2^{+} C57BL/6J (CD44$^{+/+}$) and CD44$^{-/-}$ GM-CSF BM cultures and 3x10^{5} cells were injected into the lung of BoyJ mice (CD45.2^{-}) at a 1:1 ratio. **A**, After 24 h or 1 wk, the CD44 expression and HA binding within the CD45.2^{+} donor cells in the BAL were analyzed by flow cytometry. **B and C,** Percentage **(B)** and number **(C)** of CD45.2^{+} CD44$^{+/+}$ and CD44$^{-/-}$ cells detected in the BAL after 24 h and 1 wk. Graphs show an average of three mice at 24 h and four mice at 1 wk from one experiment ± SD, with significance indicated as ** $p < 0.01$, paired t-test.

4.3 Discussion

In this study I have identified a sub population of $CD11c^+$ cells that bind high levels of HA in murine GM-CSF BM cultures. Accumulative evidence suggests that HA binding distinguishes two $CD11c^+$ populations: 1) a macrophage-like HA binding population that expresses low to mid levels of MHC II, is proliferative and capable of self-renewal, does not mature into MHC II^{high} cells and is preferentially retained in the alveolar space, and 2) a low HA binding population that expresses mid to high levels of MHC II, does not proliferate, rapidly matures into MHC II^{high} DCs and migrates to the draining lymph node during LPS-induced lung inflammation. These results were unexpected as most studies consider all of the $CD11c^+$ MHC $II^{mid/low}$ cells immature DCs that have the potential to mature into MHC II^{high} DCs upon stimulation (Bachmann et al., 2006; Lutz et al., 1999). One of the novelties of this work is that HA binding separates the $CD11c^+$ MHC $II^{mid/low}$ population into two, identifying a population that does not mature into MHC II^{high} DCs (Figure 4.3). Knowing GM-CSF can affect the development and maturation of several leukocytes including neutrophils, monocytes, macrophages and dendritic cells, it is perhaps not surprising to have more than one type of $CD11c^+$ cell in GM-CSF BM cultures.

Currently, GM-CSF derived macrophages (Fleetwood et al., 2007; Lari et al., 2007) and DCs (Inaba et al., 1992; Lutz et al., 1999) are mainly defined by their adherence to the cell culture dish, with macrophages being the more adherent population. However, this may not be the best way to distinguish between macrophages and DCs as a study by Li and Lu demonstrated that both adherent and non-adherent cells in the GM-CSF BMDC culture were able to uptake soluble antigen, and were both capable of activating T cells in a allogenic mixed lymphocyte reaction (Li and Lu, 2010). Furthermore, the adherent and non-adherent populations both expressed CD11c, and had a range of MHC II expression, although there were clearly more

MHC II^{high} cells in the non-adherent portion (Li and Lu, 2010). Preliminary data shows that there are both high and low HA binding CD11c cells in the adherent portion, although the HA binding population is enriched for (data not shown). Perhaps macrophages (HA binding) and immature DCs (non HA binding) undergo an adherent phase and as the DCs mature they become non-adherent and are able to activate T cells. Indeed, fewer T cells undergo proliferation when co-cultured with HA binding cells compared to the non HA binding MHC II^{mid} and MHC II^{high} cells (Dahiya, A. unpublished observations), suggesting HA binding cells are less efficient at T cell activation compared to the classical DCs. There are many similarities between the non-adherent HA binding CD11c^{+} and GM-CSF derived macrophages. *In vitro*, similar to the adherent macrophages, the non-adherent HA binding CD11c^{+} cells also express surface markers such as CD11b, F4/80 and CD115 (Figure 4.2A) (Lutz et al., 1999). *In vivo,* alveolar macrophages are dependent on GM-CSF for their development and are known to express high levels of CD11c (Guth et al., 2009; Vermaelen and Pauwels, 2004) and bind HA (Culty et al., 1992), similar to the HA binding population in GM-CSF BMDC cultures. Since the surface area for cells to adhere is limiting in a culture dish, it is also possible that some adherent cells become non-adherent due to the lack of space. It has become more difficult to define what is a macrophage and what is a DC due to their overlapping phenotype and function as well as heterogeneity within each cell type (Geissmann et al., 2010a). There has been a controversy between what is a macrophage or DC (Geissmann et al., 2010a; Hume et al., 2013; Randolph and Merad, 2013) and the boundaries between a macrophage and DCs are no longer clear. Here I have found that HA may help distinguish these populations in the presence of GM-CSF. Where these two lineages diverge is not clear, but literature suggests DCs and macrophages arise from a common macrophage/ DC progenitor (CMP) that generates myeloid progenitors (MDP) that generate

monocytes or common DC progenitors (CDPs), that can develop into pDCs or pre-DCs that give rise to cDCs (Geissmann et al., 2010b; Merad et al., 2013). Perhaps monocytes and pre-DCs both respond to GM-CSF and give rise to different subtypes of macrophages and DCs. Here the HA binding cells are referred as a macrophage-like population because they have more functional features in common with a macrophage than a DC, with a specific focus on their lack of ability to mature into MHC II^{high} DCs or migrate *in vivo*.

In addition to GM-CSF, the cytokine Flt3L is also an essential regulator of DC development during homeostasis, particularly in lymphoid organs (Hashimoto et al., 2011). BM cells differentiated in the presence of Flt3L produced a larger MHC II^{high} population (Xu et al., 2007), whereas BM cells differentiated in the presence of GM-CSF consistently gave rise to a larger intermediate MHC II population and a smaller MHC II^{high} population (Inaba et al., 1992; Lutz et al., 1999; Xu et al., 2007). In this study, the $CD11c^{+}$ MHC $II^{mid/low}$ HA binding population was also much larger than the MHC II^{high} population and this is presumably because the HA binding cells proliferate but do not give rise to MHC II^{high} DCs. When Flt3L and GM-CSF plus IL-4 derived BMDCs were compared directly, both were able to activate T cells *in vitro*, but GM-CSF and IL-4 derived DC were better retained in the skin, less migratory and produced higher levels of TNFα and NO (Xu et al., 2007). These results show that the GM-CSF plus IL-4 derived DCs have more macrophage-like characteristics than Flt3L derived DCs, and this may be due to the higher number of the HA binding macrophage-like cells in GM-CSF BM cultures. We are currently investigating if the $CD11c^{+}$ HA binding cells are the major producers of TNFα and NO and are also assessing their ability to activate T cells to better understand the function of this population.

The removal of endogenous HA with HAase treatment promoted the maturation of low HA binding BMDC maturation and limited the proliferation of the HA binding macrophage-like population (Figure 4.5). For a long time macrophages are thought to be replenished by monocytes (Shi and Pamer, 2011; Swirski et al., 2009), but more recent research has demonstrated the ability of macrophages to proliferate and self-renew locally (Aziz et al., 2009; Hashimoto et al., 2013; Schulz et al., 2012). The regulation of macrophage local proliferation is an area of active research and results from this study suggest that HA may play a role in this process. While the addition of HAase negatively affects the proliferation of the HA binding population, the mechanism for this remains unclear. The HAase effect could be due to the removal of HA from the cell or due to the generation of HA fragments (Shimada and Matsumura, 1980). A number of studies have provided evidence that fragmented HA can induce an inflammatory response (Jiang et al., 2007; Taylor and Gallo, 2006), stimulate the production of inflammatory mediators by macrophages and DCs, as well as induce the maturation of DCs (Jiang et al., 2005; McKee et al., 1996; Termeer et al., 2002; Termeer et al., 2000; Tesar et al., 2006). The signaling mechanism of HA fragments has been suggested to be dependent on TLR-4 and/or TLR-2 downstream signaling pathways (Jiang et al., 2005; Taylor et al., 2007; Tesar et al., 2006). While CD44 was dispensable in the activation of peritoneal macrophage with 135 kDa HA fragments from serum of patients with adult respiratory distress syndrome to upregulate chemokine *Cxcl2* gene expression (Jiang et al., 2005), CD44 was necessary for the activation of peritoneal macrophages with HA from human umbilical cord with various sizes up to 500 kDa to produce IL-6 and TNFα (Muto et al., 2009). The role of CD44 and TLR-2 or TLR-4 in mediating HA fragment induced inflammatory responses is still unclear. Perhaps CD44 is necessary for regulating certain genes but not others, or CD44 is only involved with HA of a

certain size. In this study, HAase induced DC maturation in a CD44 independent manner, however, whether CD44 is needed to interact with endogenous HA to mediate proliferation of the HA binding population remains to be examined.

Expression of hyaluronidases *Hyal-1*, *Hyal-2* and *Hyal-3* has been detected in GM-CSF BMDC cultures (Mummert et al., 2002). Hyal-2 is expressed on the surface of the cell and generates fragments of approximately 20 kDa (~50 disaccharide units). Hyal-1 is a lysosomal enzyme that degrades HA down to tetrasaccharides. Little is known about Hyal-3 (Stern et al., 2006). Degradation of HA by Hyal-1 and Hyal-2 was dependent on CD44 in human embryonic kidney 293 (HEK 293) cells (Harada and Takahashi, 2007). Another study showed that the addition of HMW HA (>570 kDa) to a day 4 GM-CSF BMDC cultures induced the expression of activation markers CD40, CD80 and CD86 independently of CD44 (Do et al., 2004). Since DCs were incubated for 48 h, perhaps they were able to generate HA fragments and DC maturation was stimulated by those fragments. Currently, little is known about the degradation of HA by DCs. Here, I identified $CD11c^{+}$ populations with different HA binding abilities and expression of surface HA. Whether these populations have different roles in HA turnover requires further investigation.

When equal number of $CD44^{+/+}$ and $CD44^{-/-}$ MHC $II^{mid/low}$ $CD11c^{+}$ cells were instilled into the lung, CD44 provided an advantage in the alveolar space, where more $CD44^{+/+}$ cells were recovered from the BAL after 24 h (Figure 4.8). The MHC $II^{mid/low}$ population consists of both the high and low HA binding cells. Due to the lack of HA binding in the $CD44^{-/-}$ cells and no cell surface marks to identify the HA binding population exclusively, I cannot be sure that there were equal numbers of $CD44^{+/+}$ and $CD44^{-/-}$ macrophage-like cells being instilled. CD44 and HA binding may have a role in the differentiation of CD11c populations in GM-CSF BMDC cultures

such that there are less macrophage-like cells in the $CD44^{-/-}$ MHC $II^{mid/low}$ population. Perhaps, it was the lack of this macrophage-like population rather than HA binding that resulted in a reduced number of $CD44^{-/-}$cells retained in the lung. Further investigation *in vivo* is necessary to establish a more definitive role of CD44 dependent HA binding in myeloid cell retention in the lung.

In conclusion, GM-CSF is a cytokine of many functions and while it promotes the differentiation of various CD11c expressing populations, I have found that only a sub population bound substantial amounts of HA. Although it is still not clear why certain cells bind HA but not others in the presence of the same cytokine, I have identified a role for CD44 in facilitating cell retention in the alveolar space, which correlated with HA binding. Finally, in contrast to the low HA binding cells, the HA binding population is highly proliferative and removal of HA limits their proliferation. This raises the interesting possibility that endogenous HA production may facilitate the self renewal of tissue macrophages. Myeloid cells are essential regulators of an immune response, a better understanding of the mechanisms by which GM-CSF and HA regulates proliferation and macrophage/DC differentiation could provide useful insights that will help generate suitable populations to target specific diseases.

Chapter 5: The lung environment promotes CD44 dependent hyaluronan binding by macrophages and CD44 provides macrophages with an advantage in the alveolar space

5.1 Introduction and rationale

HA accumulation occurs at sites of inflammation and tissue injury (Jiang et al., 2007; Stern et al., 2006) and persistent HA accumulation correlates with several diseases including; atherosclerosis (Cuff et al., 2001), autoimmune disease (Cuff et al., 2001; Tesar et al., 2006), lung fibrosis (Teder et al., 2002), asthma (Sahu and Lynn, 1978), pulmonary hypertension (Dentener et al., 2005; Ormiston et al., 2010; Papakonstantinou et al., 2008), fever (Olsson et al., 2011) and cancer (Misra et al., 2011; Toole, 2004). Together, evidence from both animal models and human patients suggests that the removal of HA fragments and restoration to the right amount of full-length HA is necessary for proper resolution of inflammation. In particular, emerging data suggest that imbalance in HA turnover may lead to the onset and progression of various pulmonary diseases (Lennon and Singleton, 2011).

In the lung, HA is distributed around the blood vessels and peri-alveolar space (Cheng et al., 2011; Ormiston et al., 2010). Alveolar macrophages are the only tissue macrophages documented with the ability to bind HA at steady state (Culty et al., 1992; Culty et al., 1994). Using an anti-CD44 antibody, Culty et al. demonstrated a role for CD44 in the uptake and degradation of HA by alveolar macrophages (Culty et al., 1992). In a bleomycin induced lung inflammation model, CD44 on hematopoietic cells was necessary for the clearance of HA, and in mice that lacking CD44, 75% died of unremitting inflammation with high levels HA accumulating in the lungs (Teder et al., 2002). These results support a role for CD44 in the clearance of HA *in vivo*, but whether alveolar macrophages were mediating this process was not

examined. In a rat model of bleomycin induced lung inflammation, increased alveolar macrophage number and macrophage motility correlated with elevated levels of HA in the lung (Savani et al., 2000). Therefore, the breakdown of HA during inflammation may allow macrophages to gain motility. The CD44 and HA interactions have been implicated to have a role in immune cell migration and HA clearance at the sites of lung inflammation (Johnson and Ruffell, 2009; Ponta et al., 2003; Pure and Cuff, 2001; Taylor and Gallo, 2006). However, the regulation and function of CD44 mediated HA binding in alveolar macrophages *in vivo* is not well understood.

Macrophages exhibit considerable plasticity, with great phenotypic and functional heterogeneity, which is often influenced by signals in their surrounding environment (Gordon and Taylor, 2005; Hashimoto et al., 2011; Mosser and Edwards, 2008; Stout and Suttles, 2004). *In vitro*, LPS plus IFNγ can stimulate the upregulation of CD11b, F4/80 and the downregulation of CD115, whereas IL-4 upregulates CD11c (Table 3.1 and Figure 3.1). *In vivo,* the lung environment changes the phenotype of peritoneal macrophages from $CD11c^-$ to $CD11c^+$, or more like alveolar macrophages (Guth et al., 2009). GM-CSF is essential for the development of alveolar macrophages (Guth et al., 2009; Paine et al., 2001; Shibata et al., 2001; Stanley et al., 1994). In chapter 4, I showed that in GM-CSF BMDC cultures, GM-CSF induced HA binding in a sub-population of cells that were $CD11c^+$ MHC $II^{mid/low}$ and exhibited features similar to macrophages. These HA binding GM-CSF derived $CD11c^+$ cells were selectively retained in the alveolar space, and CD44 provided a retention advantage (Figures 4.7 and 4.8). GM-CSF is necessary for macrophage development in the lung and HA is tightly regulated in the lung. Alveolar macrophages are the only known tissue macrophages to bind HA. Therefore, the lung provides an ideal environment to study the function of HA binding by macrophages *in vivo*. Here,

I investigate the role of CD44 mediated HA binding in alveolar macrophages, with a focus on the regulation and function of HA during inflammation. It is my hypothesis that the lung, being a GM-CSF rich organ, will maintain and induce macrophage HA binding, and this will facilitate alveolar macrophage retention in the lung.

5.2 Results

5.2.1 Alveolar macrophages constitutively bind hyaluronan, but macrophages in the lung, spleen and peritoneal cavity do not

Alveolar macrophages have been shown to bind HA (Culty et al., 1992; Culty et al., 1994). To examine if macrophages in other organs and cavities have the ability to bind HA, total cells in the BAL, the lung tissue after the BAL was collected, the peritoneal cavity and the spleen isolated from $CD44^{+/+}$ and CD44 deficient mice were labeled with Fl-HA to measure their ability to bind HA. Only cells collected from the alveolar space were able to bind high levels of HA but not cells in the lung interstitium, spleen or peritoneal cavity (data not shown). Macrophages in the lung and alveolar space are $CD11c^{+}$ $F4/80^{low}$, while the peritoneal macrophages are $F4/80^{+}$ $CD11b^{+}$ $CD11c^{-}$ and red pulp macrophages are $F4/80^{+}$ in the spleen. Although CD11c in the lung is not exclusive to macrophages (as it is also expressed on DCs), it is a useful marker to include all macrophages in the lung. $CD11c^{+}$ macrophages and DCs were gated in the lung and BAL, and F4/80 was used to identify peritoneal and splenic macrophages to examine the HA binding ability of different tissue macrophages (Figure 5.1A, top panel).

Consistent with the literature, $CD11c^{+}$ cells in the BAL, which consists of mainly alveolar macrophages (over 95% of the $CD11c^{+}$ cells in the alveolar space are alveolar

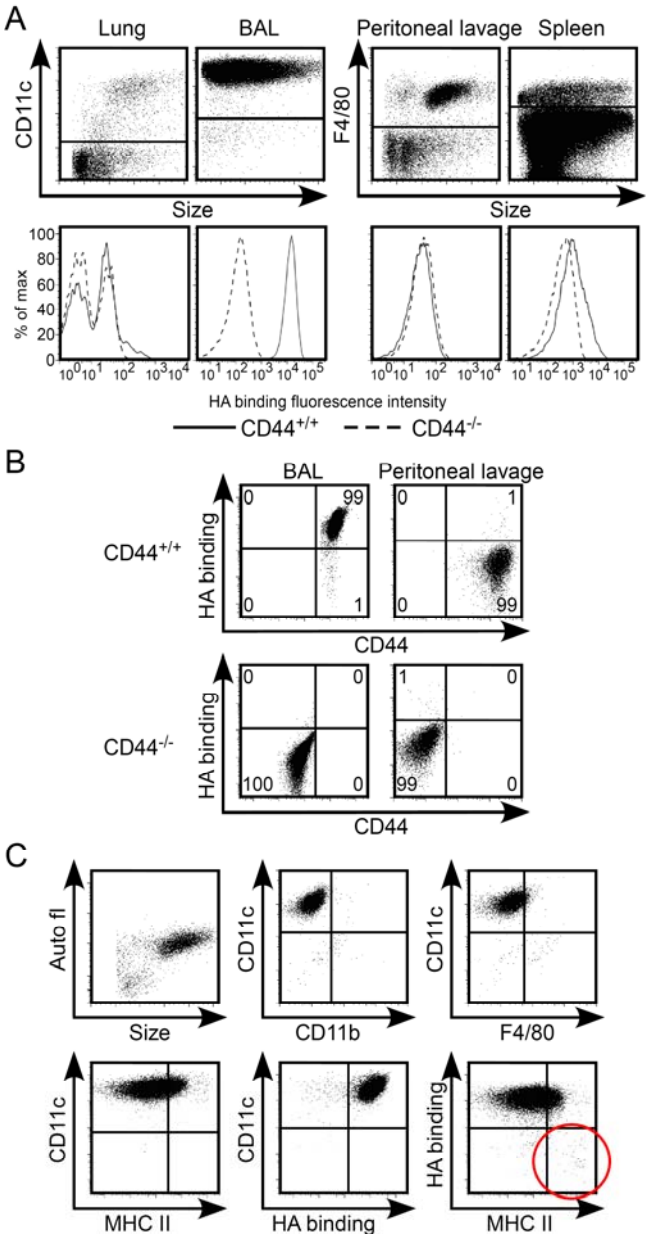

Figure 5.1 Alveolar macrophages bind high levels of HA but macrophages in the spleen, peritoneal cavity and lung tissue do not

A, $CD11c^{+}$ macrophages and DCs in the lung and alveolar space (BAL), $F4/80^{+}$ peritoneal and splenic macrophages were gated by flow cytometry (top panel, C57BL/6J mice). Macrophages from C57BL/6J ($CD44^{+/+}$, solid line) and $CD44^{-/-}$ (dotted line) mice were analyzed for HA binding by flow cytometry (lower panel). Lung tissue was processed after the BAL was collected. **B**, $CD11c^{+}$ cells were isolated from the alveolar space (BAL) and peritoneum (peritoneal lavage) of $CD44^{+/+}$ and $CD44^{-/-}$ mice, and the expression of CD44 and HA binding were analyzed by flow cytometry. **C**, Profile of HA binding alveolar macrophages collected from the BAL of $CD44^{+/+}$ mice. Circle highlights MHC II high, low HA binding DCs in the BAL. Lung, BAL and peritoneal cavity results were repeated at least three times. The spleen data was observed in six mice, examined over two experiments, three mice per experiment.

macrophages) bound high levels of HA. Interestingly, in contrast to the $CD11c^+$ cells in the BAL, only a small population of $CD11c^+$ cells in the lung were able to bind low levels of HA. Peritoneal macrophages did not bind HA. The $F4/80^+$ splenic macrophages were able to bind HA, but only at low levels. HA binding was dependent on CD44, as alveolar macrophages collected from CD44 deficient mice did not bind HA (Figure 5.1A, lower panel). These results show that tissue macrophages differ in their ability to bind HA. Specifically, $CD11c^+$ cells in the alveolar space, which are mostly alveolar macrophages, bind high levels HA, but macrophages in the lung interstitium, spleen and peritoneal cavity do not.

To determine if macrophages did not bind HA because they did not express the HA receptor CD44, the expression of CD44 in peritoneal macrophages and $CD11c^+$ alveolar cells were analyzed by flow cytometry. In the BAL, 99% of the $CD11c^+$ cells expressed CD44 and were HA binding, with 1% that expressed CD44 but did not bind detectable levels of HA (Figure 5.1B). In the peritoneal cavity, 99% of the $F4/80^+$ macrophages expressed CD44 but did not bind HA (Figure 5.1B). These results indicate that HA binding was dependent on CD44, but CD44 expression alone was not sufficient to bind HA, and HA binding was likely regulated through CD44 modifications.

The majority of the cells in the BAL were autofluorescent alveolar macrophages, with high expression of CD11c and little expression of CD11b and F4/80 (Figure 5.1C). Alveolar DCs are also $CD11c^+$ and have been distinguished from the alveolar macrophages by low autofluorescence, $CD11b^+$ $F4/80^-$ and higher MHC II expression (Vermaelen and Pauwels, 2004). Indeed, within the $CD11c^+$ population, there were two MHC II populations, the MHC $II^{mid/low}$ alveolar macrophages, and the MHC II^{high} alveolar DCs (Figure 5.1C). Analysis of CD11c expression and HA binding showed that while most of the $CD11c^+$ cells were HA binding, there

was a small population that did not bind high level of HA. Further phenotypic analysis revealed the HA binding cells as the $CD11c^+$ MHC $II^{mid/low}$ alveolar macrophages, while the $CD11c^+$ MHC II^{high} alveolar DCs bound little HA (Figure 5.1C, circled).

5.2.2 The alveolar space environment induces peritoneal macrophages to bind hyaluronan and express CD11c

In an earlier study, Guth *et al.* demonstrated how the lung environment shapes the phenotype of macrophages by instilling peritoneal macrophages that normally do not express CD11c into the lung and showed an upregulation of CD11c expression on these cells after 7 days (Guth et al., 2009). To determine if HA binding is a phenotype macrophages acquire in the alveolar space, $F4/80^+$ macrophages were purified from GFP+ peritoneal cells, then, 8×10^5 GFP^+ macrophages were placed into the lung via intratracheal instillation. The HA binding ability and phenotype of these cells were analyzed 2 weeks later. Before instillation, the $F4/80^+$ macrophages expressed high levels of GFP were $CD11b^{high}$ $CD11c^-$ and did not bind HA (Figure 5.2). After 2 weeks, cells from the BAL were analyzed and a small but clear GFP^+ population was present, indicating that some peritoneal macrophages were retained in the alveolar space. While these GFP^+ peritoneal macrophages still remained CD11b high, they became $CD11c^+$ and gained the ability to bind HA (Figure 5.2). The expression of CD11c and HA binding of the GFP^+ peritoneal macrophages was comparable to the GFP^- resident alveolar macrophages, highlighting the plasticity of macrophages, and shows that the alveolar space promotes macrophage HA binding. It was also noted that CD11b expression on GFP^- resident alveolar macrophages (Figure 5.2) was slightly higher than alveolar macrophages collected from the BAL of an untreated animal (Figure 5.1C). The increase in CD11b expression was not observed when mice were instilled

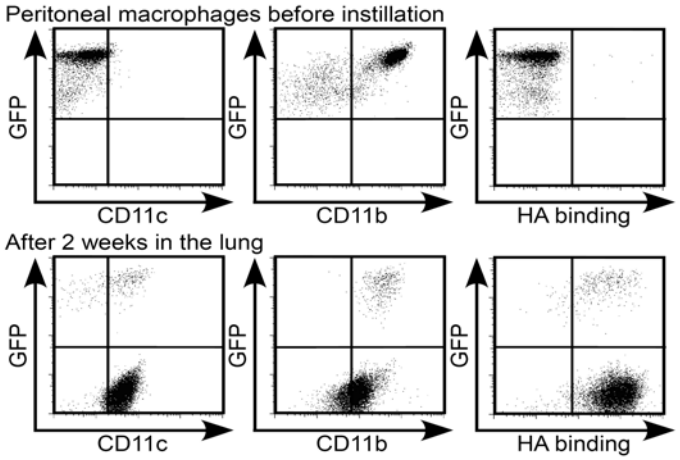

Figure 5.2 The alveolar space environment induces peritoneal macrophages to bind HA
$F4/80^+$ peritoneal macrophages were isolated and purified from GFP^+ mice and instilled into the lung. The HA binding, CD11c and CD11b expression in GFP^+ peritoneal macrophages were analyzed before instillation and after 2 weeks in the lung by flow cytometry. GFP^+ peritoneal macrophages in the lung were collected by BAL. The data shown is from one mouse, representative of six mice examined over two experiments.

with PBS solution (data not shown), suggesting the slight increase in CD11b expression was due to the addition of peritoneal macrophages (Figure 5.2). Overall, the alveolar space environment not only promotes the expression of CD11c on macrophages, but also induces HA binding.

5.2.3 GM-CSF induces hyaluronan binding in macrophages

The role of GM-CSF in the development of alveolar macrophages has been well documented (Guth et al., 2009; Paine et al., 2001; Shibata et al., 2001; Stanley et al., 1994). Here I wanted to determine if GM-CSF stimulates macrophages to bind HA. First, I examined the effect of GM-CSF on M-CSF BMDMs that have a phenotype similar to peritoneal macrophages. $CD11c^{low}$ $CD11b^{+}$ $F4/80^{+}$ and low HA binding M-CSF BMDMs were cultured in the presence or absence of GM-CSF for 48 h. M-CSF derived macrophages cultured with GM-CSF bound more HA and expressed higher levels of CD11c, while CD11b and F4/80 expression was not affected (Figure 5.3A). A similar experiment was performed on *ex vivo* peritoneal macrophages. $F4/80^{+}$ peritoneal macrophages were cultured with or without GM-CSF for 72 h. Although the effect of GM-CSF was less dramatic compared to M-CSF BMDMs, GM-CSF also stimulated HA binding and upregulated the expression of CD11c on peritoneal macrophages (Figure 5.3B). Supporting the hypothesis, GM-CSF induced HA binding and CD11c expression on both BMDMs and peritoneal macrophages and this was consistent with the phenotype observed when peritoneal macrophages were instilled into the lung. Perhaps, GM-CSF in the lung regulates and maintains alveolar macrophage CD11c expression and HA binding. GM-CSF is essential for alveolar macrophage development and since it stimulates macrophages to bind HA, it suggests HA binding may be important in alveolar macrophage development or function.

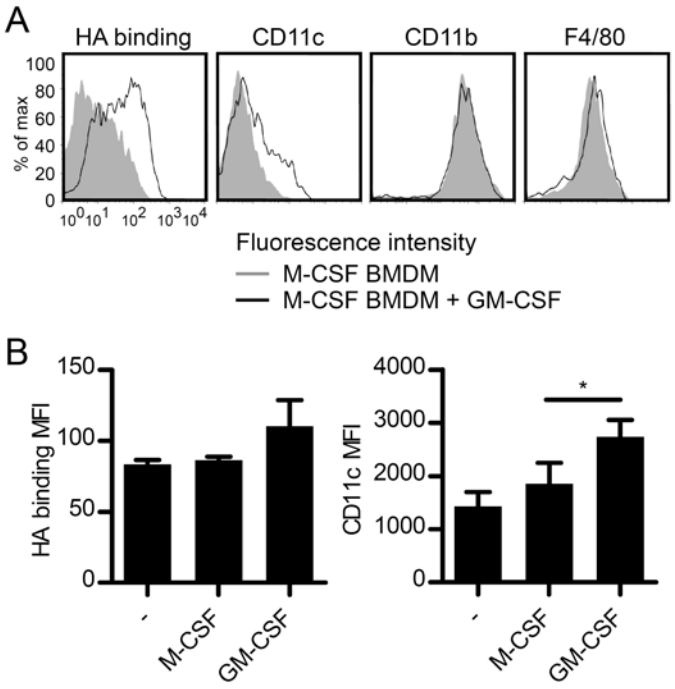

Figure 5.3 GM-CSF induces macrophage HA binding and CD11c expression
A, M-CSF derived BMDMs were cultured in the presence (black line) or absence (shaded) of GM-CSF for 48 h. Their HA binding and CD11c, CD11b, F4/80 expression were analyzed by flow cytometry. Data shown is a representative, repeated two times. **B**, F4/80$^+$ peritoneal macrophages isolated from the peritoneum of C57BL6/J mice were cultured in the presence or absence of GM-CSF for 72 h. HA binding and CD11c expression were analyzed by flow cytometry and the mean fluorescence intensity was graphed. The graphs show the average +/- SD of three mice from one experiment, repeated two times. Significance indicated as *$p<0.05$.

5.2.4 LPS reduces the number of hyaluronan binding cells during lung inflammation but these are restored upon resolution of inflammation

To begin to understand the function of HA binding by alveolar macrophages, I first examined the regulation of HA binding in the alveolar space during LPS-induced inflammation. Animals 7-9 weeks of age were given 25 μg of LPS (approximately 1 mg/kg) via intratracheal instillation. In this acute model of inflammation, animals undergo rapid weight loss and begin to recover after 4 days, which was indicated by a gradual weight gain (Figure 5.4A). The inflammatory response was also reflected in the cell numbers obtained from BAL, with the peak of cell infiltration at day 3, and cell clearance occurring by day 7 (Figure 5.4B). Interestingly, while the total number of cells in the BAL increased at day 1 (Figure 5.4A), there was a significant decrease in the number of HA binding cells at day 1 (Figure 5.4C). On the other hand, there were more HA binding cells in the BAL at day 7 compared to untreated mice (day 0). Here, it was clear that HA binding cells was being down regulated in LPS-induced lung inflammation, and restored upon resolution.

To better characterize the cells that were HA binding, the phenotype of cells in the BAL was examined at day 1, 3 and 7 post LPS injection by flow cytometry. Prior to LPS stimulation (Day 0), 97% of the cells in the BAL were HA binding. At day 1 and day 3, which were the onset and peak of inflammation, the proportion of HA binding cells reduced to 17% and 11% respectively, and then increased to 76% on day 7 (Figure 5.4D). The drop in percentage of HA binding cells can be explained by the influx of the larger $Gr1^+CD11b^+$ monocytes and small $Gr1^+CD11b^+$ neutrophils (data not shown), but it does not explain the reduced number of HA

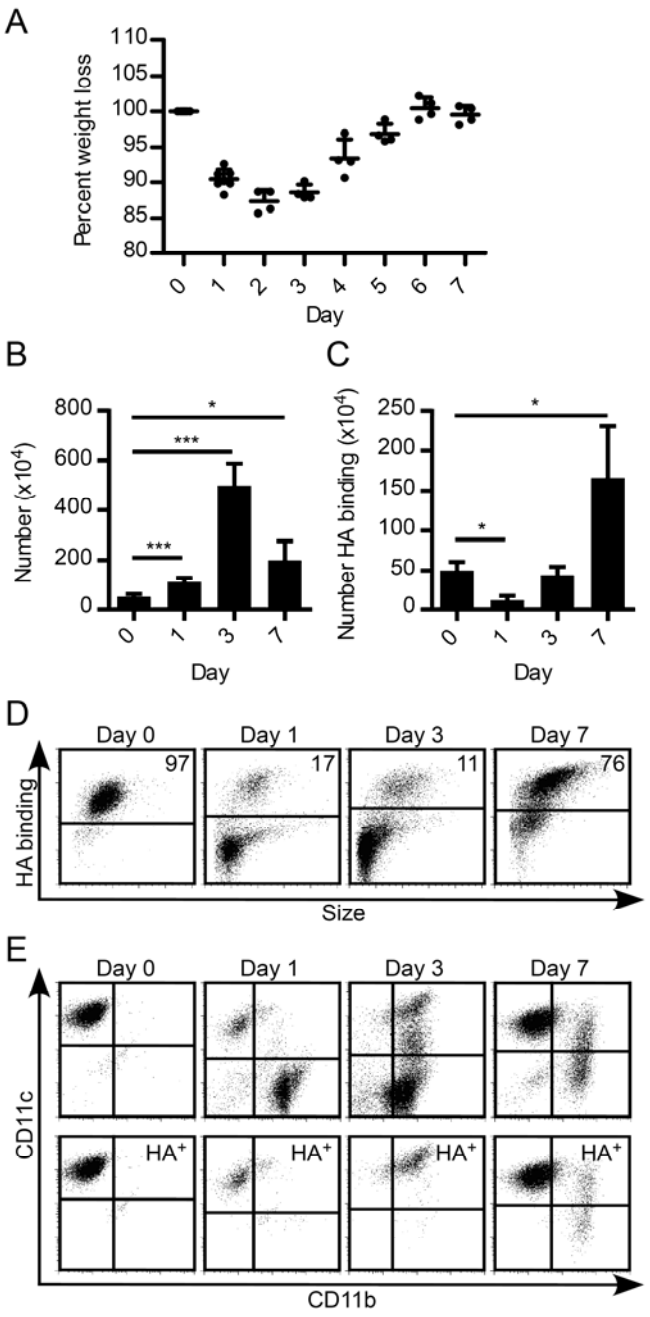

Figure 5.4 HA binding during LPS-induced lung inflammation
C57BL/6J mice were challenged with 25 μg LPS via intratracheal instillation, BAL was collected after LPS treatment at the indicated time points. **A**, Percentage weight loss, with the weight before instillation set to 100%. Graph is an average +/- SD of at least four mice from one experiment, repeated two times. **B and C,** Number of total cells **(B)** and HA binding cells **(C)** in the BAL. Graph is an average +/- SD of five mice from one experiment. This is a representative experiment repeated three times. **D and E**, Percentage of HA binding cells in the BAL analyzed by flow cytometry **(D)**. **E**, The expression of CD11c and CD11b in all the cells (top panel) and the HA binding cells (gated in **D**, bottom panel) in the BAL. This is a representative experiment repeated three times.

binding cells in the BAL (Figure 5.4C). More detailed phenotypic analysis of the HA binding population in the BAL showed that under steady state, the majority of the cells were $CD11c^+$ $CD11b^-$ and all of the HA binding cells were $CD11c^+$ $CD11b^-$ (Figure 5.4E). At day 1, there were two populations, $CD11c^+$ $CD11b^-$ and $CD11c^-$ $CD11b^+$; only the $CD11c^+$ cells were able to bind HA (Figure 5.4E). On day 3, three populations were present in the BAL, $CD11c^{high}$ $CD11b^+$, $CD11c^{mid}$ $CD11b^+$ and $CD11c^-$ $CD11b^+$; and only the cells with high CD11c expression were able to bind HA (Figure 5.4E). By day 7, most of the cells were again $CD11c^+$ $CD11b^-$ similar to day 0, and a smaller population of $CD11b^+$ cells with an intermediate CD11c expression. Here, the majority of the HA binding cells were $CD11c^+$ $CD11b^-$ similar to the phenotype of resident alveolar macrophages.

Taken together, HA binding cells were regulated in LPS-induced lung inflammation where both the percent and number HA binding cells were reduced during the early stages of inflammation and restored upon the resolution of inflammation (Figure 5.4C and 5.4D). HA binding was associated with high CD11c expression throughout inflammation. Interestingly, the phenotype of the HA binding cells shifted towards $CD11b^+$ at day 1, and were clearly $CD11c^+$ $CD11b^+$ by day 3 (Figure 5.4E). Whether this was because HA binding resident macrophages upregulated CD11b expression upon LPS stimulation or infiltrating $CD11b^+$ monocytes gained CD11c and HA binding cannot be distinguished at this point.

5.2.5 Alveolar macrophages constitutively bind hyaluronan but change phenotype during LPS-induced lung inflammation

The HA binding population in the BAL was reduced at the peak of inflammation and then increased upon recovery. Next, I wanted to determine if the decrease in HA binding cells was due to alveolar macrophages downregulating HA binding, or conversely whether the cells binding HA were no longer in the alveolar space because they migrated or died. To address this, alveolar macrophages were collected from the BAL of GFP^+ mice, then instilled into the lung of C57BL/6J GFP^- mice. GFP^+ alveolar macrophages were given 24 h to settle in the host alveolar space, and then LPS was instilled into the lungs. Cells were collected from the BAL after 1 or 7 days post LPS treatment to analyze changes in GFP^+ alveolar macrophage cell number and their ability to bind HA during an immune response. At day 1, GFP^+ cells were HA binding, but instead of being $CD11c^+$ $CD11b^-$, they expressed higher levels of CD11b (Figure 5.5A). Although the GFP^- host population did not upregulate CD11b to the same extent, some of the GFP^- $CD11c^+$ cells also became more positive for CD11b (Figure 5.5A). 7 days after LPS instillation, the GFP^+ alveolar macrophages remained HA binding, but did not express very much CD11b (Figure 5.5A). Therefore, upon LPS-induced inflammation, the phenotype of alveolar macrophages changed, with slightly increased CD11b expression, but they did not down regulate HA binding. This shows that the reduced number of HA binding alveolar macrophages during inflammation (Figure 5.4C) must be due to the loss of HA binding macrophages rather than a change in macrophage HA binding ability.

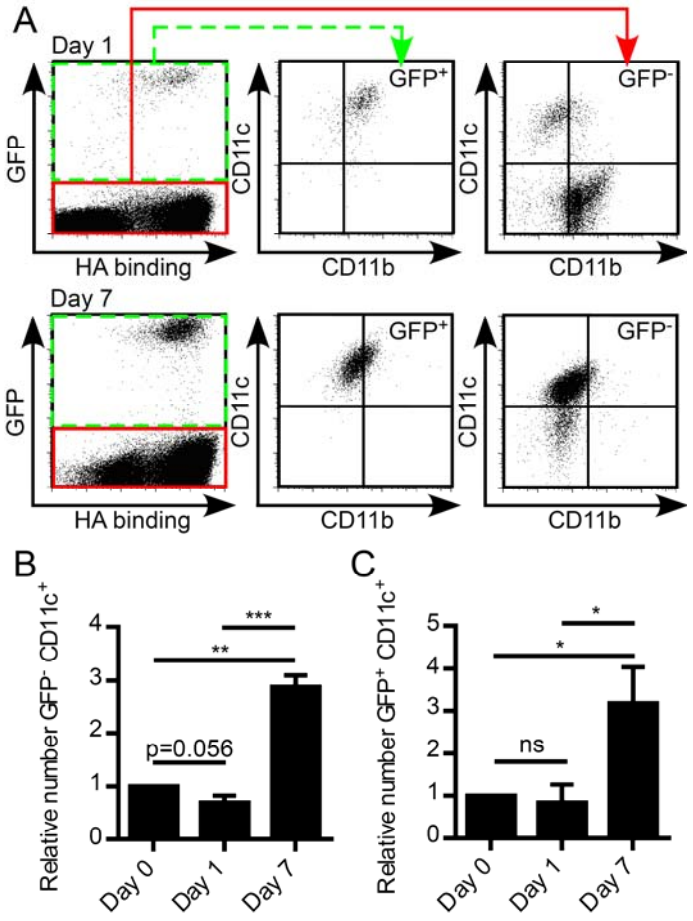

Figure 5.5 Alveolar macrophages remain capable of HA binding and proliferate during LPS-induced lung inflammation

Alveolar macrophages collected from the BAL of GFP^+ mice were instilled into the lung of C57BL/6J mice. 24 h later, mice were challenged with 25 μg LPS via intratracheal instillation and the BAL was harvested after 1 or 7 days. **A**, HA binding and phenotype GFP^+ donor and GFP^- host cells in the BAL 1 day (top) and 7 days (bottom) post LPS challenge analyzed by flow cytometry. **B and C**, Relative number of host GFP^- $CD11c^+$ cells **(B)** and the number of GFP^+ $CD11c^+$ alveolar macrophages **(C)** in the BAL. The average number of cells in the BAL 24 h after the GFP^+ alveolar macrophages were instilled, without LPS treatment was set to 1. Data shown as a mean +/- SD of three mice from one experiment, repeated two times. Significance indicated as * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

5.2.6 Hyaluronan binding alveolar macrophages proliferate during inflammation

Consistent with the earlier data (Figure 5.4C), the number of host GFP^- $CD11c^+$ HA binding cells was reduced 24 h after LPS stimulation and significantly increased 7 days later (Figure 5.5B). A similar trend was observed in GFP^+ alveolar macrophages that were instilled into the lung. Although the decrease in the number of GFP^+ alveolar macrophages at day 1 was not significant, there was significantly more GFP^+ macrophages in the alveolar space 7 days post LPS stimulation (Figure 5.5C). This indicates a loss of HA binding alveolar macrophages in the alveolar space during the early stages of inflammation. On day 7, there was 3 times more GFP^+ alveolar macrophages in the BAL compared to day 0, and also a significant increase from day 1, indicating that the alveolar macrophages must have proliferated (Figure 5.5C). In fact, both the host and GFP^+ alveolar macrophages showed a 3 fold increase in the number of $CD11c^+$ HA binding cells on day 7, which means that the host and the GFP^+ alveolar macrophages had proliferated equally (Figure 5.5B and C). Overall, these results show that alveolar macrophages do not downregulate their HA binding during LPS-induced lung inflammation, but they do upregulate CD11b expression 24 h post stimulation. Furthermore, the decrease in number of HA binding cells at day 1 post LPS treatment was due to a reduction in HA binding alveolar macrophages in the lung, and the gain in HA binding cells in the BAL at day 7 was a result of macrophage proliferation. This is the first demonstration that alveolar macrophages can self-renew after an inflammatory insult induced by LPS. Prior to this work, it was thought that recruited monocytes were responsible for replenishing these tissue macrophages.

5.2.7 Reduced number of alveolar macrophages in CD44 deficient mice

In the lung, HA is detected around the blood vessels and alveolar spaces (Cheng et al., 2011). So far I have shown that 1) alveolar macrophages constitutively bind HA, 2) non HA binding macrophages were induced to bind HA when instilled into the lung; and 3) during an immune response, the HA binding population was reduced then restored upon resolution of inflammation. These results suggest that HA binding is more important during steady state, and led to the hypothesis that HA binding facilitates macrophage retention or survival in the alveolar space and there would be less cells in the alveolar space of $CD44^{-/-}$ mice, which do not have the ability to bind HA. Supporting the hypothesis, there were significantly fewer cells in the BAL of $CD44^{-/-}$ mice compared to $CD44^{+/+}$ mice (Figure 5.6A). Then, to determine if the difference in cell number only occurs at sites where cells bound high levels of HA, we then examined the number of cells in the lung and spleen (Figure 5.6 B and C) where little or no HA binding was detected (Figure 5.1A). To analyze the number of cells in the lung, the BAL was first collected to remove cells in the alveolar space, and then the lung was perfused to remove circulating blood cells from the lung. Interestingly, there was no significant difference in the total number of cells in the lung (data not shown) or the number of leukocytes identified by CD45 mAb in the lungs of $CD44^{+/+}$ and $CD44^{-/-}$ mice (Figure 5.6B). Similar to the lung, there was also no difference in the number of cells collected from $CD44^{+/+}$ and $CD44^{-/-}$ spleens (Figure 5.6C). Here, CD44 only affected the number of cells in the alveolar space where they bound HA, and not in the lung or spleen where cells did not bind high levels of HA. These results suggesting that CD44 mediated HA binding and not CD44 alone may have a role in cell retention or survival in an HA rich areas, like the alveolar space.

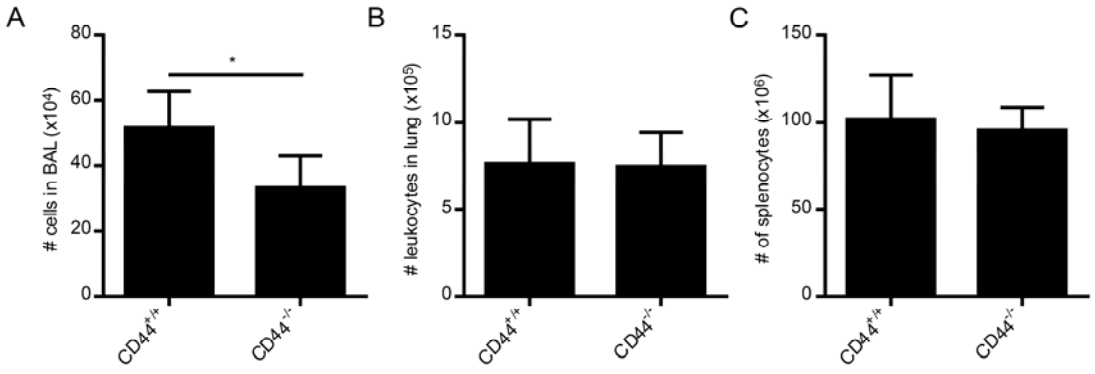

Figure 5.6 CD44 deficient mice have reduced number of cells in the alveolar space
A, Total number of cells collected from the BAL of C57BL/6J ($CD44^{+/+}$) and $CD44^{-/-}$ mice. **B**, Number of leukocytes, identified by CD45 mAb isolated from three right lobes of $CD44^{+/+}$ and $CD44^{-/-}$ mice after the BAL was collected. **C**, Total number of cells in the spleen of $CD44^{+/+}$ and $CD44^{-/-}$ mice. The BAL and lung data were from the same mice, graphs show an average +/- SD of five mice from one experiment, repeated three times. Data shown for the spleen is an average +/- SD of three mice from one experiment, a representative of two experiments. Significance indicated as * $p < 0.05$.

5.2.8 CD44 provides alveolar macrophages with an advantage in the alveolar space

More macrophages obtained from the BAL of $CD44^{+/+}$ mice compared to $CD44^{-/-}$ mice provided a positive correlation between HA binding and cell retention or survival in the alveolar space, but it does not specify whether CD44 was needed in the alveolar macrophages. To determine if it was an instrinsic or extrinsic effect of macrophage CD44 in enhancing the number of macrophages in the BAL, $CD44^{+/+}$ and $CD44^{-/-}$ alveolar macrophages were instilled into the lung to determine if CD44 conferred a competitive advantage in retaining the alveolar macrophages in the lung. Equal numbers of $CD44^{+/+}$ and $CD44^{-/-}$ alveolar macrophages that express the congenic marker CD45.2 were instilled into $CD45.2^{-}$ BoyJ mice, and the BAL was analyzed 7 days later to determine if CD44 provides macrophages with an advantage within the same host. 99% of the cells instilled were positive for CD45.2, and within the $CD45.2^{+}$ cells there were approximately equal proportions of $CD44^{+/+}$ and $CD44^{-/-}$cells, with 53% $CD11c^{+}$ $CD44^{+/+}$ and 47% $CD11c^{+}$ $CD44^{-/-}$ alveolar macrophages. All of the $CD44^{+/+}$ cells were able to bind high levels of HA while the $CD44^{-/-}$ cells did not bind HA (Figure 5.7A). 3×10^{5} alveolar macrophages were placed into the lung via intratracheal instillation. 7 days later, some of the injected alveolar macrophages were retained in the alveolar space as 2.4% of the cells collected from the BAL were $CD45.2^{+}$. Within the $CD45.2^{+}$ population, there was an increased proportion of $CD44^{+/+}$ cells, with 69% $CD11c^{+}$ $CD44^{+/+}$ HA binding alveolar macrophages and 31% $CD11c^{+}$ $CD44^{-/-}$ non HA binding alveolar macrophages present after 7 days (Figure 5.7A). Results from three mice showed a significant increase in the percentage of $CD44^{+/+}$ alveolar macrophages in the BAL compared to $CD44^{-/-}$ alveolar macrophages (Figure 5.7B). These results show that CD44 in alveolar macrophages provides an advantage in the alveolar space.

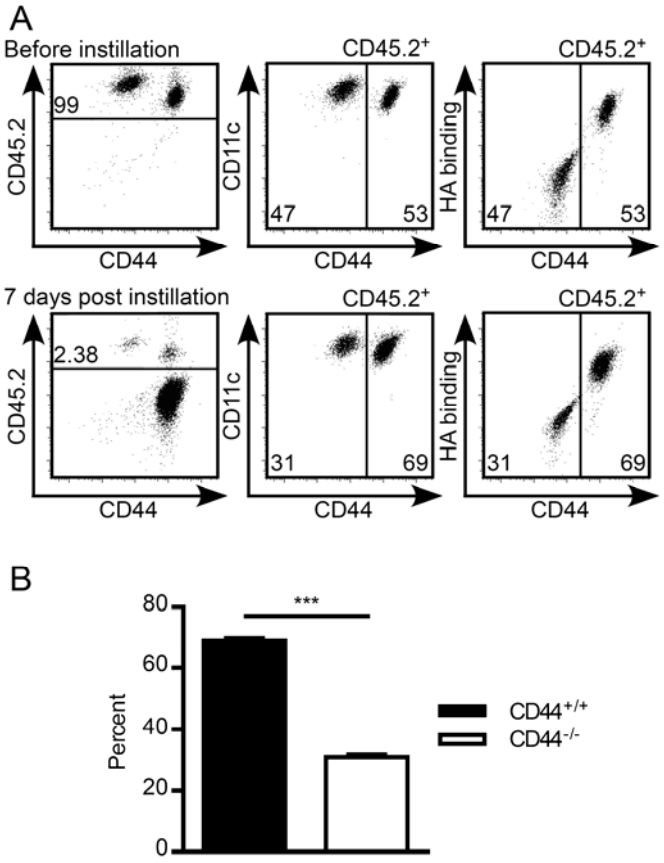

Figure 5.7 CD44 expressing alveolar macrophages have a competitive advantage in the alveolar space

Alveolar macrophages were collected from the BAL of C57BL/6J ($CD44^{+/+}$) and $CD44^{-/-}$ mice. 3 x 10^5 $CD45.2^+$ alveolar macrophages with 1:1 ratio of $CD44^{+/+}$ and $CD44^{-/-}$ macrophages were instilled into the lung. **A**, The profile of macrophages before and after 7 days in the lung were analyzed by flow cytometry. The instilled macrophages were identified by $CD45.2^+$ (first column), and CD44 expression. CD11c expression and HA binding within the $CD45.2^+$ population in the BAL were further analyzed. **B,** Percentage of $CD44^{+/+}$ and $CD44^{-/-}$ cells within the $CD45.2^+$ gated cells in the BAL after 7 days. Graphs show an average of three mice from one experiment ± SD, repeated twice. Significance indicated as *** $p < 0.001$, paired t-test.

5.2.9 CD44 plays a role in the repopulation of alveolar macrophages to the alveolar space upon competitive bone marrow reconstitution

To further investigate the role CD44 plays in macrophage recruitment/ retention/ renewal in the alveolar space, $CD44^{+/+}$ and $CD44^{-/-}$ competitive BM repopulation experiment was performed. This experiment allows $CD44^{+/+}$ and $CD44^{-/-}$ hematopoietic stem cells to differentiate into alveolar macrophages in the same environment with the only the difference being CD44 expression on hematopoietic cells. $CD44^{+/+}$ BM cells expressed both congenic markers CD45.1 and CD45.2, where as $CD44^{-/-}$ BM cells only expressed CD45.2. $CD44^{+/+}$ and $CD44^{-/-}$ BM cells were mixed at a 1:1 ratio and 8 million of the mixed BM cells were intravenously injected into lethally irradiated BoyJ mice which were $CD45.1^{+}$ $CD45.2^{-}$. Cells were harvested from the BAL after 7 weeks. Similar to the the previous results (Figure 5.7), there were more $CD44^{+/+}$ cells that repopulated the alveolar space than $CD44^{-/-}$ cells, indicated by 75% $CD45.1^{+}$ $CD45.2^{+}$ alveolar cells that originated from $CD44^{+/+}$ BM cells compared to 20% $CD45.1^{-}$ $CD45.2^{+}$ alveolar cells that originated from $CD44^{-/-}$ BM cells and only 3% $CD45.1^{+}$ $CD45.2^{-}$ host cells, which was expected due to irradiation (Figure 5.8A). Phenotypic analysis showed that both $CD44^{+/+}$ and $CD44^{-/-}$ BM cells, as well as the host BM cells recruited to the alveolar space, developed into $CD11c^{+}$ alveolar macrophages and expressed similar levels of CD11c, suggesting no defect in the differentiation process. Furthermore, as expected, only the host and $CD44^{+/+}$ $CD11c^{+}$ cells were able to bind HA (Figure 5.8B). Quantitative analysis of multiple mice indicated significantly more $CD44^{+/+}$ cells in the alveolar space compared to $CD44^{-/-}$ cells (Figure 5.8C).

Since monocytes have been shown to replenish alveolar macrophages after lethal irradiation (Landsman and Jung, 2007; Maus et al., 2006), the proportion of $CD44^{+/+}$ and $CD44^{-/-}$

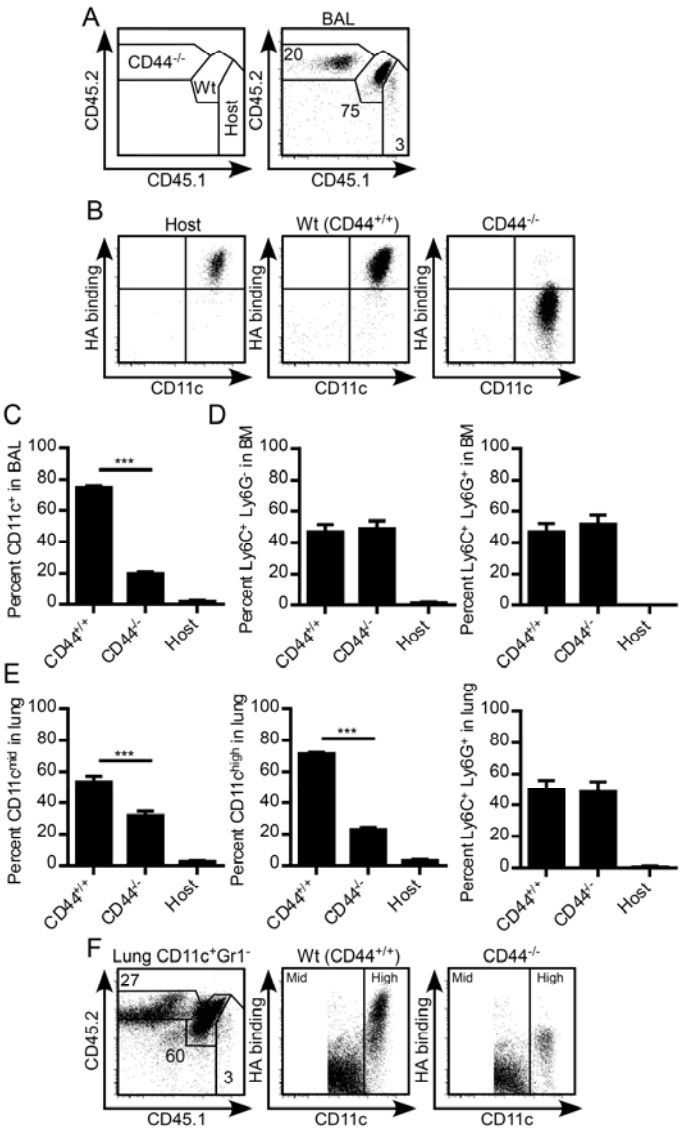

Figure 5.8 Enhanced number of CD44$^{+/+}$ CD11c^{+} cells repopulated the lung following competitive bone marrow reconstitution

BM cells were isolated from C57BL/6J/ BoyJ heterozygote (CD44$^{+/+}$, CD45.1^{+} CD45.2^{+}) and CD44$^{-/-}$ mice (CD45.1^{-} CD45.2^{+}). 8 x 10^{6} BM cells with 1:1 ratio of CD44$^{+/+}$ and CD44$^{-/-}$ cells were injected intravenously into BoyJ mice (CD45.1^{+} CD45.2^{-}) irradiated with two doses of 650 Rads. BM, lung and the BAL were harvested seven weeks after reconstitution. **A**, Proportion host, CD44$^{+/+}$, CD44$^{-/-}$ cells in the BAL analyzed by their CD45.1 and CD45.2 expression. **B**, HA binding and CD11c expression in the host, wildtype (WT, CD44$^{+/+}$) and CD44$^{-/-}$ cells in the BAL as gated in **A**. **C**-**E**, Proportion of CD44$^{+/+}$, CD44$^{-/-}$ and host cells within a specific cell population. **C**, CD11c^{+} cells in the alveolar space (BAL). **D**, Monocytes (Ly6C^{+} Ly6G^{-}) and neutrophils (Ly6C^{+} Ly6G^{+}) in the BM. **E**, CD11c^{mid} Gr1^{-}, CD11c^{high} Gr1^{-} cells and neutrophils (Ly6C^{+} Ly6G^{+}) in the lung after the BAL was collected. **F**, Proportion of host, CD44$^{+/+}$, CD44$^{-/-}$ CD11c^{+} Gr1^{-} cells in the lung, and their CD11c expression and HA binding. Graphs show an average of six mice from one experiment ± SD, with significance indicated as *** $p < 0.001$, paired t-test. This is representative data, repeated in ten mice over two experiments.

monocytes marked by $Ly6C^+$ $Ly6G^-$ in the BM 7 weeks after reconstitution was examined. In contrast to the difference in percentage of $CD44^{+/+}$ and $CD44^{-/-}$ cells observed in the BAL (Figure 5.8C), there was no significant difference in the proportion of $CD44^{+/+}$ and $CD44^{-/-}$ monocytes or $Ly6C^+$ $Ly6G^+$ neutrophils in the BM (Figure 5.8D). Blood monocytes do not bind HA during steady state (Culty et al., 1994), and similarly, monocytes in the BM also did not bind HA (data not shown). In the lung, there were two distinct CD11c populations, a $CD11c^{mid}$ and $CD11c^{high}$ population. Examination of CD11c cells in the lung tissue revealed a greater CD44 advantage in the $CD11c^{high}$ population compared to the $CD11c^{mid}$ cells (Figure 5.8E). In contrast to the $CD11c^+$ cells, the proportions of $CD44^{+/+}$ and $CD44^{-/-}$ $Ly6C^+$ $Ly6G^+$ neutrophils were equal, demonstrating a selective CD44 advantage in $CD11c^+$ leukocytes (Figure 5.8E). Gating on the $CD44^{+/+}$ $CD11c^+$ $Gr1^-$ cells in the lung, only the $CD11c^{high}$ cells were binding HA, and not the $CD11c^{mid}$ cells (Figure 5.8F). As expected, $CD45.2^+$ $CD45.1^-$ $CD11c^+$ $Gr1^-$ cells derived from $CD44^{-/-}$ BM cells did not bind HA (Figure 5.8F). The CD11c expression, the level of HA binding as well as the relative CD44 advantage in the $CD11c^{high}$ population in the lung (Figure 5.8 E and F) was similar to the $CD11c^+$ cells in the BAL (Figure 5.8B). Perhaps, the $CD11c^{high}$ cells in the lung were alveolar macrophage that were not completely removed from the lavage or macrophages that were becoming alveolar macrophages and moving towards the alveolar space. Here, it appears that the enrichment of $CD44^{+/+}$ cells is enhanced when the cells bind HA. Overall, results from competitive $CD44^{+/+}$ and $CD44^{-/-}$ BM reconstitution demonstrated a CD44 advantage in the repopulation or maintenance of $CD11c^+$ leukocytes in the lung, with the greatest advantage observed in the $CD11c^+$ HA binding macrophages in the alveolar space.

5.3 Discussion

Here, a novel role for CD44 in maintaining the macrophages in the alveolar space was identified, and the CD44 advantage strongly correlated with HA binding. In this study, more $CD44^{+/+}$ cells remained in the alveolar space after $CD44^{+/+}$ and $CD44^{-/-}$ competition experiments using both alveolar macrophages (Figure 5.7) and BM cells (Figure 5.8). The enhancement of CD44 expressing alveolar macrophages was observed when macrophages were directly instilled into the lung, suggests that the CD44 provides a retention, survival or proliferative advantage rather than recruitment from the bone marrow. Several tissue macrophages have been examined in this study and only alveolar macrophages constitutively bound high levels of HA. In the lung, HA is located around the alveolar space and blood vessels in the lung (Cheng et al., 2011). The localization of HA supports the model that HA binding by alveolar macrophages facilitates adhesion to the extracellular lung surface. There is no documentation of HA distribution around the peritoneal cavity, or in the spleen. It would be interesting to investigate sites of HA deposition *in vivo* and examine whether macrophages in other HA rich tissues, such as the skin (Fraser et al., 1997) also bind HA. *In vitro*, GM-CSF induced M-CSF BMDMs and peritoneal macrophages to bind HA and upregulated the expression of CD11c (Figure 5.3). *In vivo*, during homeostasis, GM-CSF has been detected in the BAL, whereas no GM-CSF was detected from peritoneal lavage (Guth et al., 2009). In addition, peritoneal macrophages gained HA binding and CD11c expression after being instilled into the alveolar space for 2 weeks (Figure 5.2). From these results, I propose that GM-CSF in the lung shapes the $CD11c^{+}$ HA binding phenotype of alveolar macrophages and macrophages bind HA around the alveolar space to stay localized.

In this study, CD44 expressing $CD11c^{+}$ cells have a competitive advantage in the lung, and the CD44 advantage was most dramatic in HA binding macrophages (Figure 5.7 and 5.8).

GM-CSF and HA levels are upregulated in diseases. GM-CSF expression is enhanced in diseases (Hamilton and Achuthan, 2013; Hamilton and Anderson, 2004) such as allergen induced skin reaction (Kay et al., 1991), asthmatic lung (Bentley et al., 1993) and in tumors (Borrello et al., 2005). GM-CSF can promote the differentiation of monocyte-derived DCs (Xu et al., 2007), production of inflammatory cytokines by monocytes and macrophages (Fleetwood et al., 2007), and stimulate proliferation (Falk and Vogel, 1988). Neutralization of GM-CSF or inhibition of signaling has been found to be effective in suppressing several models of inflammatory and autoimmune diseases as well as cancer (Hamilton and Achuthan, 2013). Interestingly, HA content is enriched in tumors, and increased HA levels correlate with more malignant tumors and lower survival rates in patients (Toole, 2004). Tumor cells have been shown to induce HA synthesis by fibroblasts (Knudson et al., 1984). In several models of lung disease, the amount of HA increased during inflammation, but it is normally cleared and restored upon the resolution of inflammation (Liang et al., 2007; Teder et al., 2002). When HA is not properly removed, HA accumulation correlates with more severe lung disease. Enhanced GM-CSF and HA both seem to contribute to disease progression. A connection between HA and GM-CSF is the ability of GM-CSF to induce both monocytes (Levesque and Haynes, 1997) and mature macrophages to bind HA (Figure 5.3). Thus, increased GM-CSF during disease state may induce cells to bind to the excess HA accumulating in the tumors or at sites of inflammation and promote the retention of undesired cells. CD44 mediated HA binding might also promote the generation of inflammatory HA fragments and exacerbate disease. However, it will require reconstitution with a loss of HA binding CD44 mutant alveolar macrophage to prove that it is the HA binding capacity of CD44 that is important for retention.

I also made the finding that HA binding alveolar macrophages proliferate *in vivo* in the resolution phase of an inflammatory response induced by LPS (Figure 5.5 B and C). A recent study also illustrated the ability of alveolar macrophages self-renew, as macrophage repopulation occurred independent of bone marrow precursors or circulating monocytes (Hashimoto et al., 2013). These results complements each other and together alveolar macrophages have been shown to self-renew during homeostasis (Hashimoto et al., 2013), influenza viral infection (Hashimoto et al., 2013), as well as in Poly (I:C) (Hashimoto et al., 2013) and LPS (Figure 5.5) induced inflammation. The study by Hashimoto et al. also showed that alveolar macrophage self-renewal was dependent on GM-CSF receptor (Hashimoto et al., 2013). Since GM-CSF induces macrophage HA binding (Figure 5.3), it will be interesting to determine if HA binding promotes macrophage proliferation. When $CD44^{+/+}$ and $CD44^{-/-}$ alveolar macrophages were instilled at a 1:1 ratio, we observed a competitive advantage of $CD44^{+/+}$ cells in the alveolar space 7 days post instillation (Figure 5.7), which provides enough time for macrophages to proliferate. Therefore, CD44 advantage in the alveolar space could be due to increased retention or enhanced proliferation during this time. Similarly, in the competitive BM reconstitution study, BAL was examined 7 weeks following reconstitution, which also allows time for the reconstituted macrophages to self-renew. CD44 and HA have been implicated in promoting the proliferation of endothelial cells *in vitro* (Trochon et al., 1996; West and Kumar, 1989), providing evidence that together HA and CD44 may play a role in macrophage proliferation in the lung. CD44 clearly helps maintain alveolar macrophages in the alveolar space, in this study we have not directly examined the role of CD44 and HA binding on macrophage proliferation, therefore CD44 may have a role in macrophage retention, proliferation or both processes. Earlier time

points will need to be examined to distinguish between a role for CD44 in retention versus proliferation.

In summary, the lung environment and GM-CSF induces macrophages to bind HA. In an LPS-induced lung inflammation model, HA binding cells are reduced during inflammation and restored upon resolution, suggesting a role for HA binding in homeostasis. Together these results support a model where GM-CSF rich environments induce macrophages to bind HA in the tissue, and this facilitates macrophage recruitment/ retention/ expansion in tissues.

Chapter 6: Summary and future directions

6.1 Regulation of hyaluronan binding in macrophages and dendritic cells

Here I have shown that HA binding by macrophages and DCs is dynamically regulated in response to cytokines and their tissue environment. *In vitro*, I identified cytokines and stimuli that promote HA binding and some that do not. M1 BMDMs polarized in the presence of LPS and IFNγ had a higher binding capacity for HA compared to M2 BMDMs stimulated with IL-4. LPS plus IFNγ promoted HA binding by downregulating CS sulfation on CD44, whereas IL-4 induced HA binding to a lesser extent, and dampened the ability of macrophages to bind HA by increasing CS sulfation. This was illustrated by the significant enhancement of HA binding by M2 BMDMs after the addition of LPS and after the removal of CS from CD44 (Figure 3.2 and 3.4). Similarly, BM cells cultured in the presence M-CSF gave rise to macrophages that bound little or no HA (Figure 3.2), however when cultured in GM-CSF, there was a subpopulation that bound high levels of HA (Figure 4.1 and 4.2). HA binding is tightly regulated in both macrophages and DCs. Specifically, cytokines that can promote inflammation, such as GM-CSF and LPS plus IFNγ induced higher HA binding compared to non inflammatory cytokines M-CSF and IL-4.

GM-CSF BMDC culture was used as a model to study HA binding in DCs, surprisingly, the HA binding population had several macrophage characteristics and lacked a number of features unique to DC. The HA binding cells in a GM-CSF BMDC culture did not mature into MHC II^{high} cells, were not effective at stimulating T cell proliferation *in vitro* (data not shown), and were selectively retained in the lung tissue while the MHC II^{high} non HA binding cells migrated to the draining lymph node upon inflammation. Therefore, the HA binding cells in GM-CSF BMDC cultures are likely a macrophage-like population. Interestingly, the *in vitro* results

were recapitulated *in vivo*, as peritoneal and splenic macrophages that depend on M-CSF for maturation did not bind HA, whereas alveolar macrophages that require GM-CSF to develop, constitutively bound high levels of HA (Figure 5.1). One of the critiques that often arise from using the GM-CSF BMDC model is its relevance to DCs *in vivo* and the DC populations they represent. A recent study indicated the involvement of GM-CSF in DC development *in vivo*, specifically in the lung, skin and lamina propria (Greter et al., 2012). The phenotype and morphology of the HA binding BMDCs were very similar to that of alveolar macrophages. This raises the possibility that both macrophages and DCs are present in a GM-CSF BMDC culture, with the majority of cells being HA binding, macrophage-like cells rather than DCs, and therefore, it is difficult to identify DCs *in vivo* with a similar phenotype. Both alveolar macrophages and the HA binding BMDCs were adherent upon re-plating with a round morphology and were both $CD11c^{+}$, HA binding and MHC $II^{mid/low}$ (Figure 4.1, 4.2 and Figure 5.1). Although, BMDCs expressed CD11b but alveolar macrophages were $CD11b^{-}$. BMDCs were readily retained in the lung (Figure 4.6) and remained viable for at least 2 weeks (data not shown). Even though alveolar macrophages and the HA binding cells in GM-CSF BMDC cultures were not identical, these results demonstrated considerable similarities between the two populations. The HA binding population is approximately 50% of the $CD11c^{+}$ cells in day 7 GM-CSF BMDC cultures, and perhaps they best represent alveolar macrophages. GM-CSF BMDC cultures may give rise to both macrophages and DCs, and this explains the difficulties in finding the corresponding DCs *in vivo*. Future experiments will examine this question by directly comparing the function of alveolar macrophages and the HA binding cells from GM-CSF BMDC cultures, including their ability to produce cytokines and activate naïve T cells upon stimulation. In addition, *ex-vivo* alveolar macrophages should be cultured *in vitro* in the presence

of GM-CSF, to determine if alveolar macrophages upregulates CD11b expression when cultured under the same condition as GM-CSF BMDCs. If they are indeed similar in terms of function, then isolation of the HA binding population from GM-CSF BMDC cultures would provide a useful approach to study alveolar macrophages *in vitro*.

In vivo, high HA binding was detected in the alveolar space but not in the spleen, pleural cavity, BM or mediastinal lymph node. Non HA binding peritoneal macrophages were induced to bind HA after being instilled into the lung, demonstrating that the lung environment promotes HA binding (Figure 5.2). While alveolar macrophages bind high levels of HA, it was interesting that $CD11c^{+}$ MHC II^{high} DCs in the BAL were non or low HA binding (Figure 5.1), again similar to the *in vitro* GM-CSF BMDC cultures where the MHC II^{high} BMDCs were low HA binding. It appears that HA binding is not only regulated by cytokines like GM-CSF, but might also be regulated by in a cell type specific manner, where cells that will become MHC II^{high} (likely DCs) do not bind HA even when they are in the same GM-CSF-rich environment. In support of this, little or no HA binding was observed in organs that contain larger numbers of MHC II^{high} DCs such as the spleen (Figure 5.1) and the mediastinal lymph node (Figure 4.6). To test the hypothesis that DCs do not bind HA, it would be useful to examine HA binding in a Flt3L BMDC culture. Flt3L is known to expand DCs but not macrophages from BM and Flt3L is needed for the development of most DCs in lymphoid organs (McKenna et al., 2000). Characterization of HA binding in Flt3L BMDCs during maturation will provide further insight into the regulation and function of HA binding in DCs.

6.2 Role of macrophages and dendritic cells in hyaluronan degradation and fragmentation

M1 BMDMs took up more HMW HA whereas M2 BMDMs took up more LMW HA, suggesting that M1 and M2 macrophages respond differently to different sizes of HA (Figure 3.7). Here, I have also shown that MHC $II^{mid/low}$ cells in GM-CSF BMDC cultures express higher surface HA as detected by HABP compared to MHC II^{high} cells (Figure 4.4A), suggesting they produce different amounts of HA. Therefore, different macrophage and DC subsets may have different roles in HA turnover, but this does not provide direct evidence to show that M1, M2 BMDMs or GM-CSF BMDCs process HA differently. To address this, the generation of HA fragments and degradation of HA after feeding HMW or LMW HA to different macrophage and DC subsets can be assessed by HA ELISA, which measures the amount of HA and gel electrophoresis that will differentiate the different sizes of HA. Total HA released into the cell culture supernatant can also be measured to compare HA synthesis. Hyaluronidases Hyal-1 and Hyal-2 have been implicated in the degradation and fragmentation of HA, respectively (Stern, 2005). Determining the expression of *Hyal-1* and *Hyal-2* as well as the hyaluronan synthase genes *Has-1, Has-2, Has-3* in M1 and M2 BMDMs would provide insight into the role of inflammatory and healing macrophages in HA turnover. If M1 macrophages indeed play a role in the generation of HA fragments, they may express higher levels of *Hyal-2* compared to *Hyal-1*. GM-CSF BMDCs are known to express both *Hyal* and *Has* genes (Mummert et al., 2002), however it is not known is whether subpopulations of BMDC with different HA expression or HA binding ability would express different levels of hyaluronidase and hyaluronan synthase and it would be worth further investigation.

In vitro-derived M1 and M2 BMDMs are commonly used to study the function of inflammatory and healing macrophages. In a bleomycin induced lung inflammation model, HA accumulates in the lung and HA homeostasis is restored upon resolution (Teder et al., 2002). To determine if inflammatory macrophages actually play a role in HA fragmentation and if healing macrophages are efficient in the removal of LMW HA during inflammation, macrophages could be isolate and purify from the inflammatory and healing phases of bleomycin induced lung inflammation and then compare their ability to uptake and degrade HA.

6.3 Role of CD44 and hyaluronan binding in macrophage and dendritic cell tissue retention

In this study, three competition assays *in vivo* all supported a role for CD44 in providing macrophages with a retention advantage in the alveolar space. Competitive instillation of $CD44^{+/+}$ and $CD44^{-/-}$ MHC $II^{mid/low}$ BMDCs into the alveolar space showed a CD44 advantage (62% $CD44^{+/+}$: 38% $CD44^{-/-}$) in the BAL after just 24 h, and this difference persisted after 1 week (Figure 4.8). When $CD44^{+/+}$ and $CD44^{-/-}$ alveolar macrophages at 1:1 ratio were instilled into the lung, again there was a $CD44^{+/+}$ advantage (69% $CD44^{+/+}$: 31% $CD44^{-/-}$) after 1 week (Figure 5.7). In a competitive BM reconstitution assay, there was also a CD44 advantage (75% $CD44^{+/+}$: 20% $CD44^{-/-}$) in the BAL after 7 weeks (Figure 5.8). Interestingly, the ratio was very similar between the three models, and suggests that the CD44 advantage is a result of the same mechanism. In these studies, the CD44 advantage in the alveolar space always correlated with HA binding, but it does not mean HA binding was necessary and this warrants further investigation. The retention of macrophages by HA binding would provide a possible explanation as to the role of CD44 in macrophage recruitment to HA rich atherosclerotic lesions

(Cuff et al., 2001). The lack of HA binding may result in reduced numbers of alveolar macrophages in CD44 deficient mice, and this may lead to an insufficient number of macrophage mediated clearance of HA in the lungs after bleomycin challenge (Teder et al., 2002). Tumor associated macrophages accumulate in tumors, which are also rich in HA (Misra et al., 2011; Toole, 2004) and although the ability of tumor macrophages to bind HA is not known, it would be interesing to examine if HA binding retains pro-tumor macrophages in the tumor environment and augments tumor growth.

These results support the role of HA binding in macrophage retention, but there is a caveat to this conclusion. The CD44 effect observed in alveolar macrophages and in the BM competition assay were after 1 week, and because alveolar macrophages can proliferate in the lung after an inflammatory stimulus (Figure 5.5), the role of CD44 in homeostatic proliferation or survival cannot be excluded. However, the CD44 dependent survival or trafficking advantage was observed in BMDCs after 24 h (Figure 4.8), which supports a role for CD44 in retention rather than proliferation or survival as DCs are not expected to significantly proliferate or apoptose during this short time course. Because the CD44 advantage in BMDCs after 1 week was the same as the 24 h time point (Figure 4.8), it is possible the CD44 advantage in alveolar macrophages will also be due to retention. To demonstrate this, a 24 h $CD44^{+/+}$ and $CD44^{-/-}$ alveolar macrophage competition assay would be required.

Overall, these experiments demonstrate a CD44 advantage in the alveolar space and suggest a role for CD44 in tissue retention and this correlated well with HA binding. However, to assess the role of HA binding in tissue retention, more direct experiments are to remove the ligand, HA from the tissue with hyaluronidase treatment. In addition, the use of loss of function CD44 mutant that express CD44 but cannot bind HA would be useful to test if HA binding is

necessary for macrophage retention in the alveolar space. The first approach has been applied in a study that examines hyaluronidase as a potential therapy for lung fibrosis. Intranasal administration of bovine testicular hyaluronidase alone reduced the total number of cells in the BAL 4 h and 24 h after treatment but also led to recruitment of mononuclear cells after 48 h. Hyaluronidase treatment given eight days following bleomycin stimulation reduced the amount of TGF-β and collagen in the lung tissue, factors known to promote lung fibrosis. This suggests hyaluronidase might limit the progression of fibrosis. The mechanism by which hyaluronidase regulates fibrosis remains unclear, as it may directly act on HA and remove inflammatory HA fragments, or indirectly by regulating the recruitment of mononuclear cells with a mesenchymal stem cell phenotype into the alveolar space (Bitencourt et al., 2011). Although the effect of hyaluronidase on the number of alveolar macrophages in the BAL was not examined in the above study, the fact that hyaluronidase rapidly reduced the number of cells in the BAL supports that hypothesis that HMW HA is required to retain cells in the BAL, and that HA degradation can lead to the recruitment of immune cells. To test this hypothesis, the number of $CD11c^{+}$ HA binding alveolar macrophages needs to be measured 4 h and 24 h post hyaluronidase treatment. If HA is indeed required for macrophage retention, it is expected that the number of alveolar macrophages would decrease upon hyaluronidase treatment.

CD44 R43A mutant contains a point mutation that prevents HA binding, and using this mutant in competition with $CD44^{-/-}$ or $CD44^{+/+}$ cells will help determine whether macrophage CD44 advantage in the alveolar space is dependent on HA binding. If the CD44 advantage is due to HA binding, then in competitive assays between $CD44^{+/+}$ (wildtype) and CD44 R34A mutant, $CD44^{+/+}$ macrophages would have an advantage over CD44 R34A macrophages in the alveolar space, similar to the $CD44^{+/+}$ advantage over $CD44^{-/-}$ cells, as both CD44 R34A and $CD44^{-/-}$ cells

do not bind HA. On the other hand, if the CD44 advantage was independent of HA binding, then $CD44^{+/+}$ macrophages would not have any advantage over CD44 R34A macrophages.

6.4 Role of CD44 and hyaluronan binding in macrophage and dendritic cell proliferation

Specific myeloid cells in the tissue can proliferate locally to self-renew. Langerhans cells have been shown to self-renew in tissues (Chorro et al., 2009). Langerhans cells expand rapidly by proliferation during the first week of life, with 60-70% Langerhans cells in cycle 4 days after birth, indicated by Ki67 expression. Langerhans cell proliferation diminished during steady state, as the percentage of $Ki67^{+}$ Langerhans cells reduced to 5-7% in 14 weeks old mice (Chorro et al., 2009). One of the mechanisms that can induce Langerhans cell proliferation is the activation of keratinocytes with vitamin D (Chorro et al., 2009). However, depletion of vitamin D receptor on keratinocytes only limited proliferation induced by MC903, a vitamin D3 analogue, but did not affect steady state proliferation, suggesting other mechanisms are involved in regulating Langerhans cell proliferation during homeostasis (Chorro et al., 2009). Alveolar macrophages require GM-CSF receptor to develop and proliferate (Hashimoto et al., 2013). Interestingly, both Langerhans cells and alveolar macrophages are located in HA rich tissues. In this study, HA binding identified a proliferative population within GM-CSF BMDC cultures (Figure 4.3). Similarly, HA-binding alveolar macrophages also proliferated *in vivo* in response to LPS-induced inflammation (Figure 5.5). Therefore, HA binding appears to identify myeloid cells that have the capacity to self-renew.

It is unclear if GM-CSF-induced binding of HA to CD44 regulate myeloid cell proliferation and self-renewal. Because both alveolar macrophages and the HA-binding

population in GM-CSF BMDC cultures proliferated in the presence of GM-CSF, GM-CSF BMDCs could be a useful model to further examine the role of CD44 or HA binding in GM-CSF induced cell proliferation. First, to address if CD44 is necessary for proliferation, BMDCs can be labeled with CFSE and the extent of proliferation between $CD44^{+/+}$ and CD44 deficient cells could be compared. CFSE signal reduces as the cell proliferates, if CD44 macrophage-like cells have a proliferative advantage, then the CFSE signal would be less in the $CD44^{+/+}$ cells in comparison to $CD44^{-/-}$ cells. The absence of an HA binding population in the $CD44^{-/-}$ culture and the lack of cell surface markers that exclusively identifies the HA binding cells limits the ability to study the role of CD44 and HA binding using $CD44^{-/-}$ BMDCs. The use of gain and loss of HA binding mutants that express CD44, or CD44 antibodies and proteins such as Pep-1 that interfere with the CD44-HA interaction are alternative approaches to investigate the role of CD44 and HA binding in myeloid cell proliferation. Using a similar method, competitive instillation of CFSE-labeled $CD44^{+/+}$ and $CD44^{-/-}$ alveolar macrophages into the same host would clarify if the functional role CD44 is in alveolar macrophage proliferation *in vivo*. If both $CD44^{+/+}$ and $CD44^{-/-}$ macrophages proliferate at a similar rate in the lung, indicated by equal CSFE dilution, then the increase in proportion of $CD44^{+/+}$ macrophages in the alveolar space is likely due to retention and not proliferation.

The removal of endogenous HA by hyaluronidase inhibited the proliferation of the HA binding BMDCs in the presence of GM-CSF (Figure 4.5). Although hyaluronidase removes endogenous HA, it can also generate HA fragments that can act as an inflammatory signal. It is not clear if CD44 or different sizes of HA are involved in cell proliferation as it seems to vary depending on the cell type. In this study, HA binding BMDCs are the most proliferative cells in a GM-CSF BMDC culture. Earlier work from our lab also show that the HA binding T cells were

the most proliferative upon PMA/ ionomycin stimulation and antigen specific activation, both *in vitro* and *in vivo.* However, CD44 or HA binding was not needed in this process as both $CD44^{+/+}$ and $CD44^{-/-}$ T cells proliferated equally after PMN/ ionomycin stimulation (Maeshima, N. PhD Thesis). CD44 however has been implicated in the proliferation of endothelial cells induced by LMW HA of 12 oligosaccharide units (Trochon et al., 1996). The role of CD44 and HA in proliferation is cell-type specific, highlighting the need to specifically examine the role of CD44 and HA macrophages and DCs. LPS, a well established inflammatory stimulus, inhibited the proliferation of the HA binding population in a GM-CSF culture (Figure 4.6), which shows that these cells do not proliferate upon activation. At this point, we cannot distinguish if proliferation was inhibited due to the lack of endogenous HMW HA on the surface of DCs or in the culture media, or the presence of pro-inflammatory HA fragments. To address this question, HA of specific sizes can be added into the culture, to determine if HMW HA promotes proliferation, whereas the addition of LMW HA inhibits proliferation. Differences between $CD44^{+/+}$ and $CD44^{-/-}$ cultures would indicate a role of CD44 in response to HA. It would be interesting to test whether LMW HA affects proliferation or promotes the maturation of MHC II^{high} DCs, which would suggest that different size HA polymers have distinct roles in myeloid cell maturation and proliferation. Perhaps interacting with HWM HA in the tissue requires CD44 to promote cell proliferation; and in the event of inflammation or tissue injury, LMW-HA induces DC maturation signals through TLRs independent of CD44, as hyaluronidase treatment promoted $CD44^{+/+}$ and $CD44^{-/-}$ BMDC maturation to the same extent (Figure 4.4).

Several surprising findings that are contrary to dogma are emerging in the field of monocyte/ macrophage/ DC research, one of which is the ability of macrophages to self-renew by local proliferation, and mechanisms that can regulate this process are still being explored. In

this study, a number of results suggest CD44 and HA may have a role macrophage proliferation. HA binding identifies a proliferative population in the presence of GM-CSF, the removal or degradation o f HA limits cell proliferation and macrophages expressing CD44 were enhanced in the alveolar space. Better understanding of how CD44 and HA regulate macrophage proliferation and ways to regulate their interaction would be useful to limit undesirable and promote favorable macrophage functions.

6.5 Concluding remarks

In this study, I have demonstrated that HA binding is tightly regulated in macrophages and DCs. Specifically, in two separate studies I found that the presence of CD44 leads to an increased number of $CD11c^+$ HA binding cells in the alveolar space using GM-CSF derived BMDCs and alveolar macrophages. The alveolar space selects for a HA binding phenotype and CD44 provides alveolar macrophages and macrophage-like BMDCs with an advantage in the lung. These results support a role for CD44-mediated HA binding in facilitating the retention of macrophages in the alveolar space. HA binding also marks a proliferative population, present in both GM-CSF BMDC cultures and in alveolar macrophages upon the resolution of inflammation. However, whether HA binding is necessary for alveolar macrophage retention or proliferation requires further examination. Furthermore, inflammatory and healing macrophages have different binding capacities towards HA and also take up different amounts HMW and LMW HA. Together, this work highlights the interaction between myeloid cells and HA, with implications in tissue retention, proliferation, maturation and HA turnover. The importance of maintaining HA homeostasis is clearly evident in various diseases, particularly in the lung. Several important questions arise from this work: How do M1and M2 macrophages regulate HA turnover? What is

the role of CD44 and HA binding in macrophage proliferation and survival in the alveolar space? Does excess HA contribute to pathology by retaining myeloid cells during the disease state? More detailed understanding of the signals that regulate HA binding and its function may provide insight into ways to regulate macrophage and DC function to promote health and ameliorate disease.

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Appendix: The anti-inflammatory effect of LL-37 on tissue macrophages

A. Introduction and rationale

LL-37 is a small cationic peptide that can also promote or reduce inflammation by acting on macrophages. LL-37 can increase the recruitment of monocytes by inducing the production of MCP-1 (Scott et al., 2002). However, LL-37 also has potent anti-inflammatory functions, suppressing the production of TNFα and IL-12 induced by microbial products. *In vivo*, LL-37 protects animals against bacteria-induced sepsis, suggesting that its anti-inflammatory function is dominant over its role in monocyte recruitment *in vivo*. The anti-inflammatory effect of LL-37 on macrophages is well recognized and to be useful as a therapeutic agent, LL-37 would need to have specific inhibitory effect on inflammatory macrophages without inhibiting the ability of healing macrophages to promote tissue repair. Currently, the effect of LL-37 on M2 macrophages or tissue macrophages is unknown. In a separate collaborative study, Kelly Brown determined the effect of LL-37 on M1 and M2 polarized BMDMs while I determined its anti-inflammatory ability on tissue macrophages *in vitro* and *in vivo.*

B. Results

B.1 LL-37 reduces TNFα production by in vitro stimulated peritoneal macrophages

The role of LL-37 in M1 and M2 macrophage function was investigated and LL-37 was found to selectively inhibit pro-inflammatory responses of M1 BMDMs without affecting M2 macrophage function (by my collaborator, Kelly Brown) (Brown et al., 2011). There was a LL-37 dose dependent effect in the inhibition of NO by M1 BMDMs; while 5 μg/ml of LL-37 reduced NO production by 57 ± 3%, 86 ± 3% inhibition was observed with 20 μg/ml of LL-37. LL-37 (20 μg/ml) dramatically inhibited LPS or LPS plus IFNγ induced TNFα and NO production by M1 BMDMs but inhibited ROS production to a much lesser extent and did not affect arginase activity in M2 BMDMs (Brown et al., 2011). Despite the knowledge we have on the effect of LL-37 on monocytes and macrophages, very little is known about the function of LL-37 on different types of tissue macrophages. Here the effect of LL-37 was further examined in peritoneal and alveolar macrophages.

Resident peritoneal cells were isolated from C57BL6/J mice, plated and stimulated *in vitro* with 100 ng/ml of LPS in the presence or absence of 20 μg/ml of LL-37. Approximately 50% of the isolated peritoneal cells were F4/80$^+$ macrophages (data not shown). Cell supernatants were collected after 2 h and analyzed by ELISA. LL-37 consistently reduced LPS-induced TNFα secretion by approximately 40% (Figure B1 A and B). To specifically examine TNFα production by the resident F4/80$^+$ peritoneal macrophages, peritoneal cells were plated in the presence of brefeldin A (to prevent secretion and capture intracellular TNFα) and cultured with LPS or LPS plus LL-37 for 2 h. The cells were labeled with an F4/80 mAb and intracellular TNFα produced by F4/80$^+$ macrophages was determined by flow cytometry. Only the F4/80$^+$ macrophages produced TNFα (data not shown). LL-37 caused a small but significant reduction

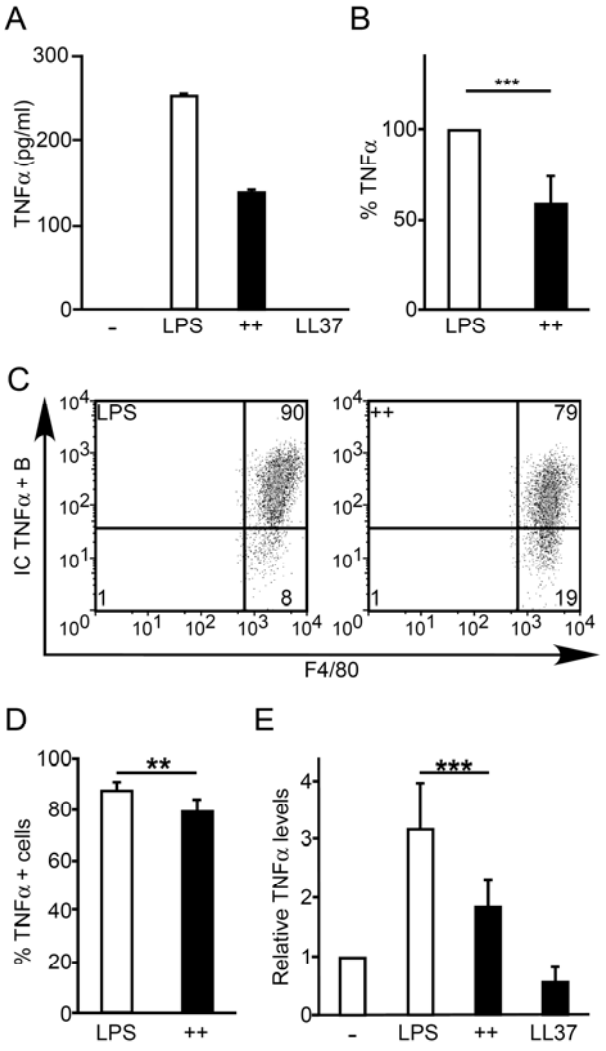

Figure B.1 Slight inhibitory effect of LL-37 on LPS-induced TNFα production in peritoneal macrophages *ex-vivo*

Cells were isolated from the peritoneum of C57BL6/J mice and 1 x 10^5 cells, 50% of which were F4/80$^+$ peritoneal macrophages, were unstimulated (-) or stimulated with 100 ng/ml of LPS (LPS), 20 μg/ml LL-37 (LL-37) or LPS and LL-37 (++) *in vitro* for 2 h, then TNFα levels in the supernatants were measured by ELISA or in F4/80$^+$ macrophages by flow cytometry. **A**, Secreted TNFα levels from one representative mouse. **B**, Average percent of TNFα produced in the presence of LL-37 (++) relative to LPS stimulation alone, which was set to 100%. **C**, Percentage of intracellular TNFα (y-axis) produced in the presence of brefeldin A (IC TNFα, +B) from LPS or LPS and LL-37 treated (++) F4/80$^+$ peritoneal macrophages (x-axis). **D**, The average percent of intracellular TNFα-positive F4/80$^+$ macrophages. **E**, Relative intracellular TNFα levels between unstimulated, LPS stimulated, LPS and LL-37 treated (++) and LL-37 treated cells. The intracellular levels of TNFα in the absence of any stimuli was normalized to 1. The ELISA data was the average +/- SD from seven mice over three experiments and the flow cytometry data was from five mice over two experiments. Significance indicated as ** $p < 0.01$, *** $p < 0.001$.

of 8% in the percent of F4/80$^+$ peritoneal macrophages that produced TNFα (Figure B1, C, and D). In addition, LL-37 reduced the levels of LPS-induced intracellular TNFα by approximately 40% (Figure B1 C and E). Thus, LL-37 modestly reduced the number of TNFα producing F4/80$^+$ peritoneal macrophages and also reduced the amount of LPS-induced TNFα secreted by resident peritoneal F4/80$^+$ macrophages over a 2 h activation period.

B.2 LL-37 reduces alveolar macrophage TNFα production in vitro

Alveolar macrophages are CD11c$^+$ and constitute the majority (approximately 90%) of cells in the BAL of C57BL6/J mice. In contrast to the mild effect of LL-37 on resident peritoneal macrophages, there was a striking inhibition of TNFα secretion by *ex vivo* alveolar macrophages that were stimulated for 2 h with LPS in the presence of LL-37. Figure B2 A shows TNFα secreted from one representative mouse and Figure B2 B shows the average percent inhibition of TNFα (90 ± 14%) by LL-37 from 8 mice. Determination of the intracellular levels of TNFα in CD11c$^+$ alveolar macrophages by flow cytometry also indicated a reduction of over 85% in the percent of cells that produced TNFα (Figure B2 C and D), and that only CD11c$^+$ macrophages made TNFα (data not shown). The level of intracellular TNFα produced by these cells was also reduced by 66% by LL-37 (Figure B2 E). Since alveolar macrophages generated nanogram per milliliter of TNFα, the addition of brefeldin A was not necessary to detect intracellular TNFα levels in these macrophages. These *in vitro* stimulated alveolar macrophages produced over 6-fold more TNFα in a 2 h period than similarly stimulated peritoneal macrophages, yet LL-37 was much more effective at inhibiting alveolar macrophage TNFα production, as assessed by the percent of cells producing intracellular TNFα (85% vs 8% reduction) and by the amount

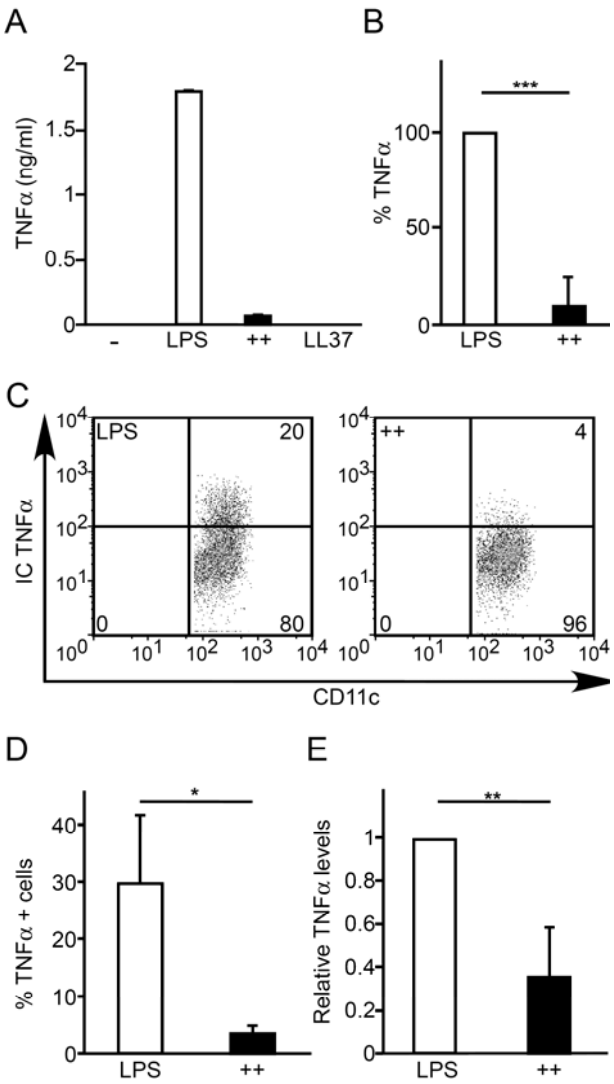

Figure B2 Inhibitory effect of LL-37 on LPS-induced TNFα production in alveolar macrophages *ex-vivo*

Alveolar cells were isolated from the BAL of C57BL6/J mice and 5 x 10^4 cells incubated *in vitro* for 2 h in the absence of stimulation (-) or stimulated with 100 ng/ml of LPS (LPS), 20 μg/ml LL-37 (LL-37) or LPS and LL-37 (++). TNFα was measured in the supernatant by ELISA or in CD11c+ alveolar macrophages by flow cytometry. **A**, TNFα levels from alveolar macrophages from one representative mouse. **B**, The average percent of TNFα produced in the presence of LL-37 (++) relative to LPS stimulation alone, which was set to 100%. **C**, Representative flow cytometry dot plots showing the percent of intracellular TNFα (y-axis) produced in the absence of brefeldin A (IC TNFα) in CD11c+ alveolar macrophages (x-axis). **D**, The average percent of intracellular TNFα positive CD11c+ macrophages. **E**, Relative intracellular TNFα levels between LPS stimulated and LL-37 treated cells where the mean fluorescence intensity (MFI) of IC TNFα from LPS stimulated cells was normalized to 1. The ELISA data was from eight mice over three experiments and the flow cytometry data was from five mice over two experiments. Error bars represent ± SD, with significance indicated as * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

of TNFα secreted (90% vs 40% reduction). Thus LL-37 significantly reduced TNFα production by LPS-stimulated alveolar macrophages *in vitro*.

B.3 *LL-37 inhibits LPS-induced TNFα production in alveolar macrophages in vivo*

To determine if LL-37 also reduced TNFα production by LPS-stimulated alveolar macrophages *in vivo*, LPS or LPS plus LL-37 were administered by intratracheal instillation into the lungs of C57BL/6J mice. After 1 h, cells and supernatants were collected from the BAL and assayed for intracellular TNFα by flow cytometry and TNFα secretion by ELISA. LL-37 dramatically inhibited (by greater than 90%) LPS-induced TNFα secretion by cells in the BAL (Figure B3 A). Only $CD11c^+$ alveolar macrophages made TNFα (data not shown) and examination of the effect of LL-37 on the intracellular levels of TNFα in the absence of brefeldin A showed that LL-37 significantly reduced, the percentage of $CD11c^+$ alveolar macrophages with intracellular TNFα by over 90% (Figure B3 B and C). This demonstrated that LL-37 significantly inhibited LPS-induced TNFα production by alveolar macrophages *in vivo.*

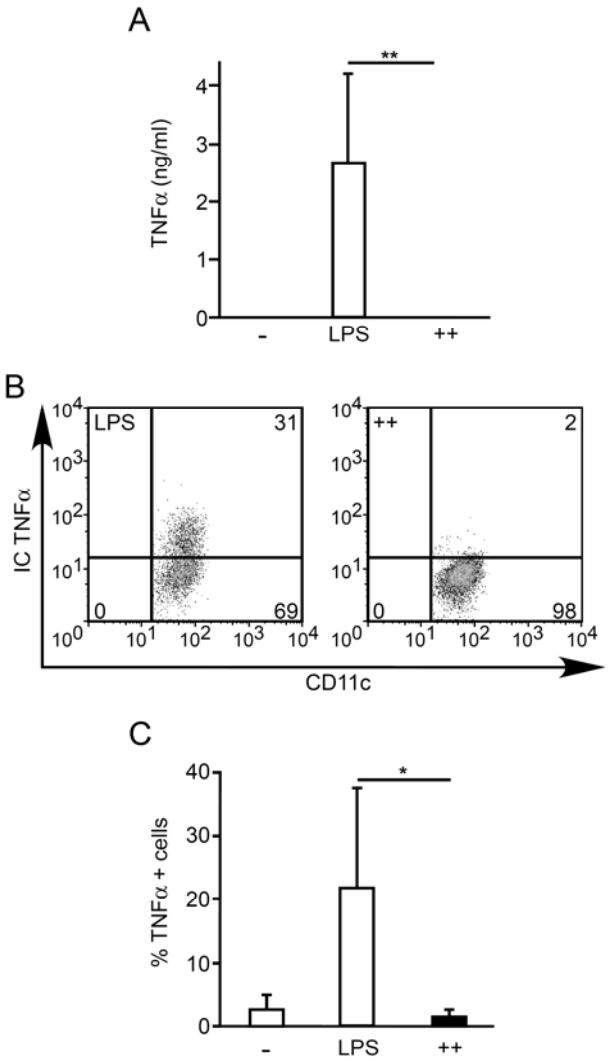

Figure B3 Inhibitory effect of LL-37 on LPS-induced TNFα production in alveolar macrophages *in vivo*

C57BL/6J mice were treated with PBS (-), 100 ng LPS (LPS), or LPS plus 20 μg LL-37 (++) by intratracheal instillation for 1 h. **A**, The average amount of TNFα present in the BAL from seven mice, measured by ELISA. **B**, Cells collected from the BAL were directly analyzed for intracellular (IC) TNFα by flow cytometry. The percent of intracellular $TNF\alpha^+$ $CD11c^+$ alveolar macrophages is shown from a representative experiment. **C**, The average percent of $TNF\alpha^+$ alveolar macrophages from unstimulated (-), LPS stimulated or LPS and LL-37 (++) treated mice. ELISA and flow cytometry data were from seven mice over three experiments. Error bars represent ± SD with significance indicated as * $p < 0.05$, ** $p < 0.01$.

C. Discussion

LL-37 exerted potent inhibitory effects on LPS-treated alveolar macrophages (Figure B2 and B3) and M-CSF BMDMs (Brown et al., 2011) but not peritoneal macrophages (Figure B1). This may relate to the phenotypically and functionally distinct nature of these two macrophage populations (Hu et al., 2004; Hu et al., 2000). Resident peritoneal macrophages are $F4/80^{+}$, $CD11b^{+}$ $CD11c^{-}$, whereas alveolar macrophages are $F4/80^{low}$, $CD11b^{-}$, $CD11c^{+}$ (data not shown). In addition, the amount of TNFα produced by the two types of macrophages in response to LPS was very different. Alveolar macrophages produced approximately 1.5 ng/ml of TNFα after a 2h stimulation *in vitro* with 100 ng/ml of LPS whereas peritoneal macrophages produced approximately 250 pg/ml. Despite this, TNFα production by alveolar macrophages was highly sensitive to treatment with LL-37, which implies that tissue macrophages have differential sensitivity to LL-37. These results highlight the functional heterogeneity between different tissue macrophages.