

**THE ROLE OF SYNDECAN-1 IN THE RESOLUTION OF CHRONIC
INFLAMMATORY RESPONSES**

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by

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THE ROLE OF SYNDECAN-1 IN THE RESOLUTION OF CHRONIC INFLAMMATORY RESPONSES

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This work, first and foremost, is dedicated to my Heavenly Father to Whom all glory belongs. This work is also dedicated to my parents, husband and son. Thank you for your unconditional love, support and patience.

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LIST OF SYMBOLS AND ABBREVIATIONS

AAA	Abdominal aortic aneurysm
AC	Adenylate cyclase
Ado	Adenosine
AKAP	A-kinase anchoring protein
ApoE	Apolipoprotein E
ATP	Adenosine triphosphate
BSA	Bovine serum albumin
cAMP	Cyclic adenosine monophosphate
CCL	CC chemokine ligand
CCR	CC chemokine receptor
CD	Cluster of differentiation
CFSE	Carboxyfluorescein succinimidyl ester
CREB	cAMP response element binding protein
CX3CR	CX3C chemokine receptor
CXCL	CXC chemokine ligand
CXCR4	CXC chemokine receptor
DAPI	4',6-Diamidino-2-phenylindole
DMEM	Dulbecco's modified eagle medium
DTH	Delayed type hypersensitivity
ECM	Extracellular matrix
EPAC	exchange protein directly activated by cAMP
FACS	Fluorescence activated cell sorting
FBS	Fetal bovine serum

FC γ R	FC gamma receptor
FGF	Fibroblast growth factor
Foxp3	Forkhead box p3
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GC	Glucocorticoids
GPCR	G-protein coupled receptor
GR	Glucocorticoid receptor
HIV	Human immunodeficiency virus
HLA-DR	Human leukocyte antigen-D related
IC	Immune Complex
ICAM1	Intercellular adhesion molecule 1
IFN- γ	Interferon-gamma
IL	Interleukin
iNOS	Inducible nitric oxide synthase
IP	Intraperitoneal
LPS	Lipopolysaccharide
LXA4	Lipoxin A4
MCP-1	Monocyte chemoattractant protein-1
MFI	Mean fluorescence index
MHCII	Major histocompatibility complex class II
MIP-1 α	Macrophage inflammatory protein-1 alpha
MMP	Matrix metalloproteinase
MR	Mannose receptor
NF- κ B	Nuclear factor kappa-light-chain-enhancer of activated B cells
NK	Natural killer

NO	Nitric Oxide
OCT	Optimal cutting temperature
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PD1	Protectin D1
PDE	Phosphodiesterase
PDGF	Platelet derived growth factor
PD-L2	Programmed death ligand 2
PGE2	Prostaglandin E2
PKA	Protein kinase A
PtdSer	Phosphatidylserine
RANTES	Regulated on activation normal T cell expressed and secreted
ROS	Reactive oxygen species
RvE1	Resolvin E1
Sdc-1	Syndecan-1
SEM	Standard error of the mean
TG	Thioglycollate
TGF- β	Transforming growth factor-beta
Th1	T helper type 1
Th2	T helper type 2
TIMP	Tissue inhibitor of matrix metalloproteinase
TLR	Toll like receptor
TNF- α	Tumor necrosis factor-alpha
Treg	Regulatory T cell
UV	Ultraviolet

VCAM1

Vascular cell adhesion molecule 1

WT

Wild type

SUMMARY

Inflammation is an integral part of the body defense mechanism that occurs in vascularized tissue in response to harmful stimuli that is perceived as being a threat to tissue homeostasis. It is a complex physiological host response that is designed to neutralize and eliminate harmful agents, initiate tissue healing, and orchestrate a return to tissue homeostasis. While inflammation is designed to be an acute event that resolves following the elimination of harmful stimuli and tissue healing, there are instances where inflammation fails to resolve and instead evolves into chronic inflammation. It is now well understood that ongoing inflammation can serve as the underlying cause of many chronic inflammatory diseases, including atherosclerosis. In fact, one of the most pressing issues that is currently faced in the field of inflammation research, one that has also become the focus of numerous ongoing investigations, is how to turn this excessive, unwarranted and undesirable inflammation response off.

Once thought to be a passive and simple process, resolution is now understood to be an active and complex process that is orchestrated by various inflammatory mediators, signaling pathways and biophysical processes. The discovery of novel biosynthetic pathways that turn on the pro-resolution signals has led to a surge in research aimed at taking a closer look at processes that can stimulate the resolution of inflammation. While major advances in the field have resulted in a better understanding of the proactive nature of resolution, many of the mechanisms involved are still unknown. To date, the repertoire of chemokine receptors that participate in macrophage clearance during resolution, for the most part, remain unidentified. Overall, there is a growing

appreciation that the discovery of mechanisms involved in the resolution responses can lead to the development of novel therapeutic approaches to resolve many chronic inflammatory diseases.

Syndecan-1 (Sdc-1), a member of a family of cell surface proteoglycans, has been previously shown to regulate events relevant to tissue repair and chronic injury responses. Sdc-1 expression during inflammation has been reported to be protective in various models, and in agreement with these published reports, our research group has recently demonstrated the protective nature of macrophages expressing Sdc-1 in the pathogenesis of abdominal aortic aneurysm. Given these observations, we hypothesize that Sdc-1 expression on macrophages is a critical component of an anti-inflammatory, pro resolution program necessary for the successful resolution of inflammatory response.

In this dissertation, we report the presence of a unique population of macrophages expressing Sdc-1 that are present within the vascular wall of mice undergoing atherosclerosis. Consistent with previous publications, the presence of Sdc-1 expressing macrophages was found to limit atherosclerosis progression. In addition, Sdc-1 expression on macrophages was associated with anti-inflammatory M2 polarization state and high intrinsic motility. Macrophage Sdc-1 expression was also linked with efferocytosis and enhanced macrophage egress from the site of inflammation to the draining lymphatic network. Moreover, we discovered that the chemokine receptor CXCR4, which was found on Sdc-1 expressing macrophages, was also involved in macrophage egress during inflammation resolution.

Following a brief overview of the overall research in Chapter 1, an attempt to provide a summary of current knowledge in the regulation of both acute and chronic

inflammation, biophysical processes involving macrophages that are believed to be important in the resolution of inflammation, and how macrophage Sdc-1 expression can potentially affect inflammation outcome and help stimulate resolution, was included in Chapter 2.

In Chapter 3, we described our initial efforts to characterize the polarization state of macrophages expressing Sdc-1. The expressions of surface markers that are known to be expressed on polarized macrophages were investigated. Additional biochemical and biophysical functions of macrophages, including the intrinsic motility of polarized and Sdc-1 expressing macrophages were investigated. These characterization efforts established the anti-inflammatory nature of Sdc-1 expressing macrophages, which was linked with increased macrophage migration response. Importantly, we demonstrated efferocytosis to be a possible regulator of macrophage Sdc-1 expression.

The correlation that we discovered between efferocytosis, Sdc-1 expression and enhanced intrinsic motility motivated us to investigate the effects of macrophage Sdc-1 expression on macrophage motility in vivo. In Chapter 4, we utilized both Sdc-1^{+/+} and Sdc-1^{-/-} mice and a murine peritonitis model to investigate the effect of Sdc-1 expression on macrophage response and egress during inflammation. Importantly, the absence of Sdc-1 was found to result in the accumulation of macrophages in the peritoneal cavity, which was associated with delayed macrophage clearance. In addition, delayed macrophage clearance was found to be associated with dampened macrophage egress toward the draining lymph nodes.

In Chapter 5, we discussed our efforts to identify specific chemokine receptor that is involved in macrophage egress during the resolution of inflammation. While we

identified 3 chemokine receptors that were upregulated in Sdc-1 expressing M2 polarized macrophages, CX3CR1, CXCR4 and CCR10, only CXCR4 was found to be expressed and to be functionally active following macrophage efferocytosis. Further tests utilizing AMD3100, a CXCR4 blocker, in murine peritonitis model confirmed that macrophage CXCR4 is involved in the regulation of macrophage response. We demonstrated that CXCR4 blockade resulted in the accumulation of macrophages within the peritoneal cavity, which was associated with delayed macrophage egress toward the draining lymph nodes. Overall, we were able to demonstrate that correlation between macrophage Sdc-1 and macrophage CXCR4 expression, and their involvement in macrophage egress toward the draining lymphatic network during the resolution of the inflammatory response.

CHAPTER 1

INTRODUCTION

1.1 Significance

Inflammation is a complex physiological host response that occurs in vascularized tissue in response to harmful stimuli that is perceived as being a threat to tissue homeostasis. It is an integral part of the body defense mechanism that is designed to neutralize and eliminate harmful agents, initiate tissue repairs, and direct a return to tissue homeostasis. While inflammation is designed to be an acute event that resolves once the harmful stimuli are eliminated, there are cases where inflammation fails to resolve. In fact, it is now understood that chronic inflammation can contribute to the pathogenesis of many diseases, including rheumatoid arthritis, autoimmune disorders, aneurysm, and atherosclerosis [1, 2]. Resolution of inflammation has traditionally been regarded as a passive process that simply involves the abatement of proinflammatory agents. However, recent findings have demonstrated that resolution is a biosynthetically active process that involves the activation of many tightly controlled biochemical mediators and signaling pathways that not only turn off the proinflammatory response, but also turn on the pro-resolution signals [1-6]. Understanding the mechanisms of the points of control governing these pro resolution signals can help uncover their therapeutic potentials, leading to the development of novel treatment approaches for many chronic inflammatory diseases, including atherosclerosis.

In this dissertation, we aim to understand the interplay between macrophage polarization state, macrophage motility and macrophage efferocytosis – three components

that are important in the resolution of inflammation – and how it relates to macrophage Syndecan-1 (Sdc-1) expression during inflammation. We found that macrophage Sdc-1 expression is associated with macrophages that display anti-inflammatory characteristics and are highly motile. In fact, efferocytosis, a process that is often considered necessary for the successful resolution of inflammation, is found to be a regulator of macrophage Sdc-1 expression both in vitro and in vivo. In addition, animal studies performed using mice lacking Sdc-1 expression in both peritonitis and atherosclerosis models suggest that the absence of Sdc-1 expression can lead to a greater inflammatory response, which is associated with dampened macrophage motility and efflux out of the inflammation site. Collectively, these results reinforce the physiological significance of macrophage efferocytosis and macrophage motility as endogenous modulators of the inflammatory response and further suggest a role for Sdc-1 in these fundamental resolution programs.

1.2 Rationale

Macrophage, a monocyte-derived cell that functions as one of the key players in inflammation, often represents the dominant leukocyte population in chronic inflammation. In fact, both early and late lesion areas in atherosclerosis consist of macrophage rich regions [7, 8]. Recent findings have brought into light the fact that not all macrophages are created equal. In fact, macrophages are made up of heterogeneous population that consists of macrophages polarized to different activation state, with distinct biochemical activity and functions [9-14]. Thus, inflammation outcome can definitely be influenced by the balance between different macrophage polarization states. Indeed, the absence of a sufficient pro resolution macrophage counterbalance may serve as a driving force in the development of chronic inflammation, including atherosclerosis.

Additional studies that look at the involvement of macrophages in atherosclerosis have also indicated that dampened macrophage efferocytosis response and increased macrophage retention in the vascular wall are both associated with plaque progression [7, 15-17]. These suggest that macrophage efferocytosis and egress are important factors that can potentially affect the resolution of inflammation.

Sdc-1, which is a member of a family of cell surface proteoglycans, has been shown to regulate events relevant to tissue repair and chronic injury responses, including cell migration and cell-substrate interaction [18-21]. The expression of syndecans has also been shown to be highly regulated by soluble growth factors that are released at the site of injury [22-25]. Our group has recently demonstrated that macrophage Sdc-1 expression was upregulated and associated with a protective effect in murine model of abdominal aortic aneurysm (AAA) [26]. Protective effects of Sdc-1 expression have also been reported in other inflammatory models, including murine model of myocardial infarction and delayed type hypersensitivity [27, 28]. Activation of signaling pathways that lead to Sdc-1 expression has also been tied to anti-inflammatory G protein coupled receptor activation [29]. Within these diverse investigations lies a common thread suggesting that macrophage Sdc-1 plays a critical part in anti-inflammatory program that leads to the resolution of inflammation. Therefore, understanding the role of Sdc-1 in dictating macrophage activation and related responses can offer new insight in the regulation of inflammation resolution and open up new venues for the development of therapeutics aimed at stimulating the resolution process.

1.3 Central Hypothesis and Specific Aims

The overall objective of this project is to understand how Sdc-1 plays a critical part in an anti-atherogenic program necessary for the clearance of monocyte derived cells out of the inflamed vascular by emigration and efferocytosis, promoting the resolution of inflammation. The central hypothesis is that Sdc-1 is a critical component of a pro-resolution program necessary for the clearance of monocyte-derived cells by emigration and efferocytosis, and its expression can promote resolution in inflammation and contribute to homeostasis. Our overall approach to test this hypothesis is to investigate the relationship and interplay between Sdc-1 expression, macrophage polarization, macrophage motility and macrophage efferocytosis both in vitro and in vivo. The specific aims for this project are: (1) characterize the role of Sdc-1 in the polarization of monocyte-derived subsets at sites of vascular lesion formation, and (2) define the role of Sdc-1 in the resolution of inflammation by promoting the emigration and/or efferocytosis of monocyte derived cells in the vascular wall.

The extent to which Sdc-1 promotes the polarization of monocyte derived cells and affects motility and/or efferocytosis has not been well defined. The outcome of this research is expected to provide significant contribution in investigating the role of Sdc-1 in influencing the clearance of monocyte derived cells out of the inflamed vascular wall and in understanding resolution processes in chronic inflammatory diseases.

CHAPTER 2

BACKGROUND

Inflammation and resolution of inflammation are two fields of research that are actively ongoing. This last decade has witnessed a profound leap in our level of understanding in the active nature of resolution process. The discovery of novel biosynthetic pathways that turn on the pro-resolution signals has led to a surge in research aimed at taking a closer look at processes that can trigger resolution and stimulate a return to homeostasis. In this chapter, we attempt to provide a summary of current knowledge in the regulation of both acute and chronic inflammation, biophysical processes involving macrophages that are believed to be involved in the resolution of inflammation, and how macrophage Sdc-1 expression can potentially affect inflammation outcome and help stimulate resolution.

2.1 Inflammation: Acute vs. Chronic

John Hunter, an 18-th century Scottish surgeon, once wrote that “Inflammation in itself is not to be considered as a disease but a salutary operation consequent to some violence or some disease” [30]. In other words, inflammation, which is a normal physiological host response to infection or tissue injury, is a protective mechanism that is designed to neutralize harmful agents, elicit tissue healing and ultimately ensure a return to homeostasis. In fact, inflammation is so life preserving that people who suffer from genetic deficiencies are associated with increased risk of severe infection that can be fatal if left untreated [31-34] . However, the term ‘inflammation’ itself often carries a negative connotation and is often considered a disease that needs to be treated. While this stems from the visible symptoms associated with inflammation: rubor, calor, dolor and tumor

(redness, heat, pain and swelling), this association most likely developed from instances where inflammation failed to resolve and turned into a prolonged event. Thus, it is important to discuss inflammation in the context of its timeframe. While acute inflammation is a healthy response that resolves within hours to days, chronic inflammation can last for weeks to months [4].

2.1.1 Acute Inflammation

By design, inflammation is supposed to be an acute event. When tissue injury occurs, tissue resident inflammatory cells of the innate immune system are activated, releasing inflammatory mediators that initiate inflammatory response. These mediators can include complement, histamine, bradykinin, and cytokines and chemokines that result in the activation of the endothelial cells and increased vascular permeability in the area of injury [35-37]. During inflammation, activated endothelial cells express adhesion molecules, such as E-selectin, intercellular adhesion molecule 1 (ICAM1) and vascular cell adhesion molecule 1 (VCAM1) that are presented on the luminal surface [38-40]. These molecules aid in the recruitment of leukocytes from the bloodstream resulting in their rapid influx into the area of injury. Neutrophils are short lived phagocytes that are among the first responders to be recruited to the inflammation site. They participate in the phagocytosis and killing of the harmful microbes and also promote the recruitment of inflammatory monocytes that mature locally into macrophages. Under normal conditions, the elimination of the harmful stimuli is followed by the apoptosis of neutrophils, which are then ingested and removed by macrophages. Effective removal of apoptotic cells from the tissue by macrophages not only prevent further recruitment of

leukocytes to the inflammation site, but also stimulate macrophage egress to the lymphatics, leading to full resolution and complete tissue healing [7].

2.1.2 Chronic Inflammation

In instances where inflammation fails to turn off and stays activated despite the absence of appropriate stimulus, chronic inflammation can ultimately result. It is now understood that chronic inflammation can contribute to the pathogenesis of many diseases, including rheumatoid arthritis, autoimmune disorders, aneurysm, and atherosclerosis [1, 2]. Unlike acute inflammation, where clearance of apoptotic neutrophils prevents further recruitment of leukocytes, chronic inflammation is characterized by the continuous influx of leukocytes to the injury site [7, 41]. Thus, chronic inflammation can result in a progressive shift of the leukocyte population to produce an inflammation that is dominated by macrophages instead of neutrophils. Activated leukocytes that accumulate at the inflammation site can also generate a lot of proinflammatory mediators, including proinflammatory cytokines and chemokines, metalloproteinases, and reactive oxygen species [7]. Overall, these factors contribute to the generation of proinflammatory feedback loop that keeps the inflammation from resolving.

While inflammation is a necessary process that serves as the body's main defense mechanism, dysregulation of the inflammatory response can result in deleterious outcome. Many check points and pathways are built into the inflammation process to ensure its resolution once the harmful stimuli are eliminated [2, 4, 6, 42, 43]. However, there are instances where these points of control become dysregulated, which then result in inflammation that progress unchecked. Thus, it is important to understand these built

in pro-resolution check points so that we can discover where they fail and how to turn on and utilize them to promote resolution.

2.2 Resolution of inflammation: molecular mechanisms

Resolution is an active and coordinated process that is equipped with both molecular and cellular mechanisms that ensure the restoration of tissue integrity and physiological function. Some of these cellular signals that are essential in the resolution of inflammation include the expression and activation of bioactive molecules, including bioactive lipid mediators, adenosine, cyclic adenosine 5'-monophosphate (cAMP), interleukin (IL)-10 and transforming growth factor- β (TGF- β), just to name a few [1-4, 6, 29, 42-48]. Others include active cellular functions of participating leukocytes that are part of the inflammatory mechanism itself, including switching/turning off of leukocyte activation state, apoptosis/efferocytosis and efflux to the lymphatic system [1, 7, 10, 16, 41, 49-51] .

2.2.1 Bioactive lipid mediators

Recent findings have demonstrated that specific omega-3 polyunsaturated fatty acid derived mediators are generated within the resolving exudates [3-6]. These include arachidonic acid derived lipoxin A4 (LXA4), eicosapentaenoic acid derived resolvin E1 (RvE1), and docosahexaenoic acid derived protectin D1 (PD1) [3-6]. LXA4 has been reported as a potent endogenous factor that can regulate leukocyte influx during acute inflammation. Specifically, LXA4 has been shown not only as a potent regulator to stop neutrophil recruitment during inflammation, but also as strong stimuli for the non phlogistic recruitment of monocytes to the inflammation site [42, 52-54]; exposure of monocyte derived macrophages to LXA4 induces rapid phagocytosis of apoptotic cells

[2, 55, 56]. Similar to LXA4, RvE1 and PD1 have both been reported to promote the clearance of apoptotic neutrophils during acute inflammation by enhancing macrophage phagocytosis and macrophage migration to the lymphatic system [3, 5, 43, 57]. RvE1 has also been reported to help inflammation resolution in a variety of disease models, including pulmonary infection, allergic airway inflammation, dermatitis, and vascular disease [43, 57-59]. In a model of pulmonary inflammation, RvE1 was reported to accelerate resolution by promoting phagocytosis induced neutrophil apoptosis [59]. RvE1 was also reported to ameliorate atopic dermatitis by suppressing leukocyte infiltration and proinflammatory cytokines production at the inflammation site [57]. Similarly, PD1 has also been reported to be protective in many inflammatory models, including asthma and acute kidney injury models [60, 61]. PD1 administration was reported to result in significant decrease in leukocytes recruitment and proinflammatory cytokines production in both models [60, 61]. Overall, these bioactive lipid mediators possess great potential as promising therapeutic target for the resolution of inflammation and are the subject of various ongoing investigations.

2.2.2 Adenosine

Adenosine, a purine nucleoside, is an endogenous anti-inflammatory agent that acts through the G-protein coupled receptor (GPCR) signaling pathway [35, 48, 62]. Biophysical activity of adenosine is entirely dependent on its concentration level and the presence of its corresponding receptor [48, 63, 64]. Thus, processes that are related to adenosine generation, release, cellular uptake and metabolism are highly regulated during inflammation. While adenosine is constitutively expressed at low concentration in the extracellular space, its expression can be upregulated in response to cellular insult or

injury [48, 63, 64]. Following its generation and release, adenosine can bind to four different types of adenosine receptors, including A₁, A_{2A}, A_{2B} and A₃, which are expressed on the surface of many types of leukocytes [48, 63, 64]. Thus, adenosine can target and regulate the function of many cells that are involved in orchestrating the inflammatory response.

Adenosine has been reported to have immunosuppressive effect on both neutrophils and macrophages. Activation of adenosine receptors on neutrophil surface – all four adenosine receptors have been reported to be expressed on neutrophils – results in reduced neutrophil trafficking and neutrophil function [62, 65-68]. As early as the 1980s, adenosine has been described to be able to suppress the generation of superoxide anion by neutrophils [62, 65-68]. Since then, adenosine has also been found to suppress other neutrophil functions, including adhesion, degranulation, and proinflammatory cytokine production and secretion [48, 69, 70]. In most cases, these protective effects associated with adenosine receptor activation are regulated through cAMP dependant pathway [48, 71].

Macrophages are another type of leukocytes that express adenosine receptors on their surface. Similar to neutrophils, macrophage adenosine receptor activation is associated with pro resolution effects on inflammation [48, 63, 64]. One of the main functions of macrophages, which are professional phagocytes, is to detect and clear harmful microbes, which can be achieved through recognition of conserved microbial elements by toll like receptor (TLR) or scavenger receptors [48, 72-76]. This leads to the generation and release of proinflammatory mediators like tumor necrosis factor-alpha (TNF- α), IL-12, macrophage inflammatory protein 1 alpha (MIP-1 α) and nitric oxide

(NO) [73-76]. At the same time, the signaling pathway that govern the expression of IL-10, a potent anti-inflammatory cytokine, is also activated [77]. However, it is important to note that IL-10 is not secreted until later in the inflammatory response time frame. One of the signals that can trigger IL-10 release from leukocytes is adenosine [77, 78]. In addition, adenosine also strongly suppresses macrophage production of proinflammatory cytokines that include IL-12 and TNF- α [79-83]. Similar to neutrophils, activation of adenosine signaling pathway is also linked to the cAMP pathway [29, 83, 84]. Overall, activation of macrophage adenosine receptor signals to macrophages that the inflammation state is shifting toward resolution.

2.2.3 cAMP

cAMP is a second messenger that lies downstream of the GPCR signaling pathway. It was discovered fifty years ago and was the first second messenger found in the field of signal transduction [85]. Since then, cAMP has been demonstrated to be a universal regulator of cellular functions that are important in the regulation of both physiological and pathological processes, including heart function, metabolism, cancer, diabetes and inflammation, just to name a few [85-89]. Macrophages are major players of the immune response that have been reported to be highly sensitive to cAMP regulation. In brief, cAMP has been reported to have anti-inflammatory and pro-resolution effect on macrophages [29, 45, 90]. Active biomolecules that have been demonstrated to increase intracellular cAMP concentration include prostaglandin E2 (PGE2) and adenosine, as discussed above [29, 84]. Thus, adenosine and cAMP shares common activation pathways that result in shared features in their regulation of macrophage physiology.

Signaling through the cAMP pathway is a complex process that involves many chemical messengers and enzymes. In brief, activation of GPCR leads to the transduction of signals which results in the activation of G proteins that activate the adenylyl cyclase (AC). AC then catalyzes the intracellular generation of cAMP from adenosine triphosphate (ATP) [45, 85]. The enzyme phosphodiesterase (PDE), which degrades intracellular cAMP, regulates the intracellular concentration level of cAMP [45, 85]. Physiological effects of cAMP are mediated by two distinct intracellular receptors, the classic protein kinase A (PKA) and cAMP response element binding protein (CREB) pathway, or the more recently discovered exchange protein directly activated by cAMP (EPAC) pathway [85, 91-95]. Together, these two cAMP signaling pathways participate in the integrated control of macrophage functions during inflammation response.

Activation of cAMP signaling pathway has been reported to inhibit many macrophages inflammatory functions. cAMP has been described to interfere with the generation of reactive oxygen species (ROS) that, while essential in microbial killing, can also result in tissue damage [45, 47, 96]. cAMP activation through the PKA pathway has also been described to inhibit the generation of proinflammatory mediators, including MIP-1 α , leukotriene B₄ and TNF- α by blocking the activation of nuclear factor kappa-light-chain enhancer of activated B cells (NF- κ B) pathway in macrophages [45, 47]. cAMP has also been found to form a multi-protein signaling complexes with A-kinase anchoring proteins (AKAPs) that result in the phosphorylation and activation of p105, a protein that can act as a cytosolic inhibitor of NF- κ B [45, 97]. Additionally, cAMP was also found to enhance the generation of IL-10 in LPS stimulated macrophages [45, 97].

Traditionally, cAMP regulation of macrophage activity has been focused on its inhibitory effects on the generation and release of proinflammatory mediators. However, the work performed by Kim et al has demonstrated that cAMP signaling pathway can also mediate macrophage migration response in anthrax model [29]. The edema factor, which is found in the anthrax toxin, was discovered to dramatically enhance the migration response of infected macrophages. Anthrax toxin was found to activate the AC, which result in intracellular cAMP increase and the activation of cAMP-PKA-CREB signaling pathway in these macrophages [29]. The enhanced macrophage migration response was found to correlate with a change in macrophage cytoskeletal structure and cell shape in general. Indeed, activation and redistribution of actin molecules, which is involved in macrophage motility, was observed in these macrophages. In addition, macrophages that were treated with 6-Bnz-cAMP, an analog of cAMP that specifically targets the PKA pathway, were found to respond in a similar manner. Activation and redistribution of actin was associated with enhanced macrophage migration following exposure to the cAMP analog [29]. Thus, cAMP-PKA signaling pathway is associated with both anti-inflammatory effects and enhanced motility response in macrophages.

2.2.4 Anti-inflammatory cytokines

While macrophages are crucial in the regulation of proinflammatory responses, they are also integral in orchestrating its resolution. Exposure of activated macrophages to apoptotic cells can trigger a shift in macrophage activation state that results in the turning off of proinflammatory mediators production and the activation of anti-inflammatory mediators that include IL-10 and TGF- β [1, 17, 98, 99]. These anti-inflammatory cytokines are important for the successful resolution of inflammation.

IL-10 is a well established anti-inflammatory cytokine with important immunoregulatory functions that can affect the functional activity of many cells that regulate the immune system [100, 101]. IL-10 expression is reported to be low in healthy tissue, but can be strongly upregulated during inflammation response. It can repress activated macrophage expression and release of proinflammatory mediators, such as TNF- α , MIP-1 α , IL-6 and IL-12 [100-102]. Macrophages that have been exposed to IL-10 can also release additional IL-10 into the microenvironment. In this manner, IL-10 can act in both autocrine and paracrine fashion to shift the global inflammatory environment toward one that is pro resolution [100, 101].

Clearance of apoptotic cells by macrophages also triggers the release of TGF- β , an anti-inflammatory cytokine that regulates many cell functions, including proliferation, differentiation and survival [103, 104]. In inflammation, TGF- β is responsible in the promotion of tissue repair and regeneration [44, 46, 105]. TGF- β can stimulate angiogenesis, the proliferation and differentiation of fibroblast, and matrix deposition through the synthesis of collagen [106-110]. It can also regulate the expression of integrins, MMPs and tissue inhibitors of matrix metalloproteinases (TIMPs), which are important in cell migration during wound healing process [46, 105, 111, 112]. Overall, these features all contribute to wound healing and the reestablishment of tissue integrity and function at the site of inflammation.

TGF- β has also been reported to regulate the activation of other immune cells during inflammation, including T cells and macrophages. TGF- β was shown to be able to suppress proinflammatory cytokine production, including IL-2 and interferon- γ (IFN- γ), which results in the inhibition of activated T cell differentiation and proliferation [104,

113, 114]. TGF- β can also induce the activation of forkhead box P3 (Foxp3), a transcriptional factor necessary for the generation of regulatory T cell (Tregs) [115, 116]. Tregs participate in the inhibition of proinflammatory cytokines generation, while also enhancing the expression of anti-inflammatory mediators, such as IL-10 and TGF- β [104, 114, 117]. In addition to inhibiting T cell activation, TGF- β can also inhibit macrophage activation. This can be attributed to TGF- β blocking of inducible nitric oxide synthase (iNOS) and MMP-12 production [118, 119]. TGF- β has also been reported to block the activation of TLR signaling pathway that is dependent on MyD88, which can participate in the production of many proinflammatory cytokines and mediators [119, 120]. Overall, the generation of anti-inflammatory mediators triggered by apoptotic cells at the site of inflammation is essential in ensuring proper wound healing and resolution of inflammatory response.

2.3 Macrophage: a key player in chronic inflammation

In addition to the secretion of bioactive molecules that regulate inflammation resolution, there are active cellular processes of leukocytes that participate in inflammatory response themselves, that are important in resolution. Macrophage, which is the main player of the mononuclear phagocyte system, is crucial in the regulation of inflammation. As early as the 1980s, Elie Metchnikoff, who won the 1908 Nobel Prize in medicine for his work on phagocytosis, has stated the importance of the phagocytes stimulation in generating immune response [13, 121-123]. Generated from the differentiation of monocytes in the peripheral tissue, macrophages are the major leukocytes that are found at the site of chronic inflammation. As mentioned previously, both early and late lesion areas in atherosclerosis are made up of macrophage rich regions

[7, 8]. In inflammation, macrophages can function as a regulator of immune response through the generation of proinflammatory mediators that contribute to the clearance and killing of harmful microbes, as a phagocyte that participates in the clearance of cellular debris and apoptotic cells, and as a promoter of inflammation resolution and tissue healing response [13, 90, 124-127]. Macrophage clearance out of the inflammation site to the draining lymphatic also signals the end of inflammation and a return to homeostasis [16, 49, 99, 128]. Thus, the regulation of macrophage activation, macrophage efferocytosis response and macrophage motility within the inflammation site are crucial in determining the outcome of inflammation.

2.3.1 Macrophage polarization state

Recent findings have shed light on the fact that not all macrophages are created equal. Macrophages are highly plastic cells that can change their physiological features in response to unique environmental cues. Microenvironmentally derived signals can give rise to macrophages that are polarized with specific functional properties [10, 13, 14, 126, 129-132]. In an effort to emulate the T helper type 1/T helper type 2 (Th1/Th2) nomenclature of the T cell literature, macrophage polarization has been classified into two extremes in the linear scale of macrophage activation, which includes classically activated, proinflammatory M1 macrophages on one end, and alternatively activated M2 macrophages on the other end [10, 13, 130]. The generic use of M2 was first utilized to describe macrophages that participate in type II immune response, tissue healing and remodeling and additionally share the common feature of having low IL-12 secretion [10, 13, 14, 130]. However, current research has demonstrated that M2 polarization represents at least three individual subtypes that function in allergy and parasite defense

(M2a), immunoregulation (M2b), and tissue repair and remodeling (M2c) [10, 13, 14, 130, 132]. Despite these classifications, given the complexity of the inflammatory environment and various cytokines that are released at the site of inflammation, it is likely that more macrophage polarization states actually exist during inflammatory response.

Classically activated M1 macrophages can be generated through the exposure of naïve macrophages to two signals, IFN- γ and an inducer of TNF (or TNF itself) [10, 13, 14]. IFN- γ is a proinflammatory cytokine that can be generated by both innate and adaptive leukocytes, including natural killer (NK) cells, activated T cells, and macrophages [14]. Exposure to IFN- γ , while necessary for priming macrophage activation, will not actually fully activate them. A second signal that can induce TNF generation is necessary to fully activate the macrophages [14]. While exogenous TNF delivered to macrophages can result in M1 activation, TNF generation is usually a result of macrophage exposure to ligands that activate the TLR signaling pathway. Exposure of macrophages to TLR ligands, such as microbes or microbial product (eg. lipopolysaccharide (LPS)), can lead to the activation of the NF κ B and MyD88 signaling pathway, which result in the generation of TNF by the macrophages themselves [14]. The secreted TNF will act in conjunction with IFN- γ in an autocrine manner to polarize macrophage to M1 activation state [14].

Classically activated M1 macrophages serve as crucial component of the host defense system against foreign pathogens in the body. M1 macrophages can be identified by their high surface expression level of major histocompatibility complex class II (MHCII) and cluster of differentiation 86 (CD86), which are essential in macrophage's

role as antigen presenting cells [10, 14]. Their biological functions are also well documented. M1 macrophages possess a markedly high level of antimicrobial activity that can be attributed to the induction of the inducible nitric oxide synthase (iNOS), which results in the generation of reactive oxygen species [10, 13]. M1 macrophages also generate high level of proinflammatory cytokines, including, TNF- α , IL-1, IL-6, IL-12 and IL-23 [10, 13, 133]. These proinflammatory mediators are essential in driving the Th1 polarization which results in efficient killing of invading intracellular pathogens and the development of tumor resistance [10, 130, 133, 134]. Indeed, studies performed using mice lacking IFN- γ expression demonstrated the importance of macrophage M1 activation state in the clearance of bacterial and viral infection [135, 136]. The lack of M1 macrophage presence in various tumor environments is also associated with tumor growth, disease progression and metastasis [133, 134, 137]. Overall, while M1 macrophages are vital to host defense system, their activation has to be tightly regulated, given the potential for excessive tissue damage to the host as a result of their secretion of proinflammatory mediators.

Early in 1990s, Gordon et al made the observation that IL4 treatment resulted in the upregulation of mannose receptor (MR) on elicited macrophages [14, 138]. The authors demonstrated that these macrophages exhibited signs of activation that was unique compared to the classically activated macrophages [138]. This unique population of IL-4 treated macrophages were described as displaying an “alternative activation phenotype” [138]. This marks the first instance in which M2 macrophages are recognized to be distinct from M1 macrophages. Since then, IL-13, another type 2 cytokines was also found to be able to induce the alternative activation of macrophages

[139-142]. IL-4 has been reported to be produced by basophils and mast cells during early phase of tissue injury response [143-145]. It has been suggested that the secreted IL-4 can quickly generated M2a macrophages, which then participate in wound healing response [146, 147]. M2a macrophages have also been found in parasitic infection, such as Helminth infection, where their existence results in dampened M1 macrophage response [139, 148-150]. While this can lead to reduced parasite killing and clearance, alternative macrophages can buffer the collateral damage impacted by M1 macrophages [139, 148, 150]. Thus, the regulation of macrophage activation during inflammation is an important feature that needs to be tightly controlled.

Studies performed on alternatively activated macrophages have demonstrated their distinct biochemical features and biological roles compared to the classically activated M1 macrophages [10, 13, 140]. IL-4 treated macrophages, which are now designated as M2a macrophages, display reduced antimicrobial activity compared to M1 macrophages. This can be attributed to their high level of arginase activity, which shares the same substrate, L-arginine, with iNOS; iNOS generates NO through the oxidation of L-arginine [151, 152]. Thus, an increase in arginase activity can result in the depletion of L-arginine, which then results in low NO production level. Similar to M1 macrophages, M2a macrophages express high level of MHCII and CD86 [10]. However, unlike M1 macrophages, M2a macrophages are not good at presenting antigen [10]. In fact, these macrophages actually inhibit Th1 response and promote the activation of Th2 response instead [133, 140, 148]. The high arginase activity of M2a macrophages has also been reported to be involved in their wound healing responses and extracellular matrix deposition [9, 153, 154]. High arginase activity can result in polyamine and proline

biosynthesis, which can result in increased production of collagen, polyamines and fibronectin, promoting tissue healing [9, 154, 155]. Additional features associated with M2a macrophages include high expression of the transcription factors, FIZZ1 and Ym1 and the upregulation of surface expression of programmed death ligand 2 (PD-L2), a Th2 stimulated molecule that inhibits T cell proliferation [12, 14, 156-159].

Another macrophage polarization state was discovered when macrophage Fc gamma receptors (FC γ Rs) activation by immune complexes (IC) unexpectedly generated macrophages that secrete low level of IL12, but high level of IL-10; FC γ Rs are receptors found on the surface of leukocytes that can bind to the Fc portion of an antibody [14, 160, 161]. These unique macrophages were referred to as type II activated macrophage, or M2b polarized macrophages. Similar to classically activated M1 macrophages, M2b macrophages require two signals for their activation, FC γ R ligation and macrophage stimulatory signals that result in cytokine production [10, 14]. This can usually be achieved through the activation of the TLR signaling pathway. Type II activated macrophages or M2b macrophages are unique compared to M1 or M2a macrophages. Compared to M1 macrophages, M2b macrophages secrete low level of IL-12, though other cytokine production, such as TNF, IL-1 and IL-6, remain similarly high [10, 14]. The surface expression level of MHCII and CD86 also remain high compared to M1 macrophage [10, 14]. Compared to M2a macrophages, M2b macrophages express low level of arginase activity [10, 14]. However, similar to M2a, M2b macrophages also induce a Th2 response [10, 133]. Another interesting feature of M2b macrophages is their high secretion of IL-10, which is an anti-inflammatory cytokine [10, 153]. An adoptive transfer study, in which M2b macrophages were transferred into mice receiving

high dose of LPS demonstrated the protective effect of M2b macrophages [162]. Overall, these studies demonstrate that antibody can serve as a signal in the regulation of the inflammatory response.

Another population of macrophage that is important in the resolution of inflammation is the regulatory macrophages, also known as M2c macrophages. M2c macrophages are anti-inflammatory, pro resolution macrophages that display reduced MHCII and CD86 surface expression [10, 13]. M2c macrophages can arise following macrophage exposure to IL-10, TGF- β or glucocorticoids (GC) [10, 140, 163, 164]. GC is a member of the steroid hormones that can be released by adrenal cells in response to stressful conditions [163, 165]. The binding of GC to glucocorticoid receptor (GR) has been shown to result both in the upregulation of anti-inflammatory protein expression and in the inhibition of proinflammatory cytokine production [163, 164]. As mentioned previously, IL-10 and TGF- β are two anti-inflammatory mediators that can be released by macrophages following exposure to apoptotic cells [1, 50, 99, 166, 167]. These cytokines can then act in both autocrine and paracrine manner to generate M2c macrophages and dampen the overall immune response.

Current research has identified four distinct macrophage polarization states in the murine which include M1, M2a, M2b and M2c polarization. Despite these classifications, it is still unknown where these distinct polarization states actually exist in vivo, or whether macrophages actually exist in a spectrum of polarization states. Given the complexity of the inflammation response and the multitude of cytokines that are released at the inflammation site, it is likely that macrophages can develop a more complex polarization state in vivo. Adding to the complexity, while distinct macrophage

polarization states can be found in human, human macrophages have been reported to exhibit differences to murine macrophages in their response to polarizing stimuli [129, 168-170]. For example, Arginase and Ym1, markers for M2a polarization state in murine macrophages, were not upregulated in human M2a macrophages [168, 169]. Nevertheless, macrophages are major players of the inflammatory process and their manipulation can prove to be key in stimulating its resolution.

2.3.2 Macrophage efferocytosis

The main function of inflammation is to eliminate harmful stimuli that are perceived as being a threat to tissue homeostasis. However, apoptotic cells that are generated in the process can be damaging to the host tissue if not removed promptly from the inflammation site [7, 50, 167, 171]. Apoptotic cells, if not promptly cleaned up, will become necrotic cells that progressively lose their cell plasma membrane integrity, resulting in cell swelling and eventually spilling of the intracellular toxic content onto the environment. Cellular debris generated can then serve as proinflammatory signals, resulting in continuous leukocyte influx into the tissue, which contribute to the maintenance of inflammation. Thus, macrophage ‘janitorial’ service that ensures the cleaning up and removal of cellular debris and apoptotic cells before they turn necrotic is highly essential to the resolution of inflammation [7, 13, 50, 167].

The term efferocytosis fittingly comes from the Latin word *effero* meaning to take to the grave or to bury [15, 166]. It is a highly efficient and immunologically silent process by which apoptotic cells are engulfed and cleared by phagocytes, which includes macrophages. While efferocytosis serves an integral part of immune response, it is also important in the day to day maintenance of homeostasis. In fact it is estimated that more

than 10^{11} of circulating neutrophils, which patrol the blood stream as a part of the body's defense system, are removed and 'recycled' through efferocytosis every single day [15]. During efferocytosis, cell membrane of the phagocytes can ruffle and protrude to engulf the apoptotic cells, forming large, fluid filled phagosomes, also known as efferosomes [15, 172-175]. Macrophages recognize apoptotic cells by absence of 'don't-eat-me' signals, which include CD47, and the expression of 'eat-me' signals, which include phosphatidylserine (PtdSer), lysophosphatidylcholine and calreticulin on their cell surface [15, 166, 176, 177]. Recognition of these signals by macrophage's tethering receptors that include CD14 and CD31 can result in the formation of phagocytic synapse. Further activation of the low-density lipoprotein receptor-related protein and PS receptor results in the activation of signaling pathway that initiates the extension of the phagocyte's membrane ruffles, leading to the total engulfment of the apoptotic cells [166, 172, 174, 175].

One of the defining features of efferocytosis is the non immunostimulatory nature of the process. On the contrary, engulfment of apoptotic cells has been shown to have an anti-inflammatory effect on macrophages. Contrary to the phagocytosis of invading pathogens, uptake of apoptotic cells does not result in the generation of proinflammatory mediators. In fact, efferocytosis has been demonstrated to have an anti-inflammatory effect on macrophages [16, 50]. As mentioned previously, macrophage exposure to apoptotic cells can trigger the secretion of anti-inflammatory mediators, including IL-10 and TGF- β [1, 50]. Efferocytosis has also been demonstrated to dampen macrophage production of proinflammatory mediators such as TNF and IL-12, following exposure to LPS [50, 166]. In fact, efferocytosis can have such immunosuppressive effects that it is

utilized in apoptotic cell-based therapy as an approach to prevent or to treat allograft rejection and graft versus host disease in transplantation [178-180].

Another study also found that efferocytosis can affect macrophage motility during inflammatory response. Utilizing a murine peritonitis model, it was found that a unique macrophage population expressing low level of CD11b was present during the resolution phase of the inflammation [99]. The CD11b^{low} macrophages display unique features compared to their CD11b^{hi} macrophage counterpart, including expressing lower level of iNOS, MMP-9 and displaying dampened inflammatory response to TLR stimulation [99]. In addition, these macrophages were found to be more likely to egress to the draining lymphatics compared to the CD11b^{hi} macrophages [99]. Significantly, CD11b^{low} macrophages were found to have engulfed more apoptotic cells compared to the CD11b^{hi} macrophages. In fact, subsequent exposure of CD11b^{hi} macrophages to apoptotic cells can result in the reduction of surface CD11b expression [99]. Overall, this suggests that apoptotic cell exposure can alter macrophage phenotype into one that is more motile and more pro resolution.

Some of the turning points in inflammatory response that signal the shift from inflammation to resolution are the apoptosis of infiltrating leukocytes and their clearance by macrophages via efferocytosis. In addition, subsequent macrophages egress from the inflammation site is essential to achieve full resolution and a return to homeostasis. This suggests that macrophage motility is potentially another factor that is important in determining the outcome of inflammation.

2.3.3 Macrophage motility: chemokine and chemokine receptor

Leukocyte migration is an important factor in the regulation of inflammation. Early in the inflammatory response neutrophils are recruited from the bloodstream into the injured tissue where they release proinflammatory factors that result in the further recruitment of inflammatory monocytes to the site. Subsequently, macrophage migration out of the inflamed tissue into the draining lymphatics is crucial in achieving full inflammation resolution. While a lot has been studied regarding the regulation of neutrophils and monocyte influx into inflammation site, not as much is known regarding macrophage migration within the tissue and macrophage efflux to the lymphatics during resolution. The recent paradigm shift whereby the active nature of resolution is recognized has led to the notion that the regulation of macrophage motility during inflammation response is not as trivial as it was once believed and can in fact be important in determining inflammation outcome.

Leukocyte migration is governed through the expression of chemokine receptors and chemokines. Chemokine receptors are superfamily of GPCRs containing seven transmembrane domains that are almost exclusively expressed on leukocytes [10, 37, 181]. Each family of chemokine receptor can bind with their corresponding family of chemokines, a group of low molecular weight proteins that serve as chemoattractant. While chemokine – chemokine receptor interaction has been reported to be involved in organ development, angiogenesis, malignancy, and human immunodeficiency virus (HIV) infection, its traditionally defined role has been to control leukocyte trafficking in immune response and inflammation [182-188].

Chemokine – chemokine receptor interaction is responsible in controlling the migration of circulating monocytes from the circulation into their target tissues. Similar

to macrophages, monocytes are also comprised of heterogeneous populations. Current research has indicated that there are at least two monocyte subsets that exist in the murine system. Resident monocytes expressing high level of CX3C receptor (CX3CR) type 1, low level of Ly-6C and no CC chemokine receptor (CCR) type 2 (CX3CR1^{hi}CCR2⁻Ly-6C^{lo}) are targeted into a variety of non-inflamed tissues where they serve as precursor to resident macrophages [189-191]. In contrary, high level of CCR2, high level of Ly-6C and low level of CX3CR1 expression on inflammatory monocytes (CX3CR1^{lo}CCR2⁺Ly-6C^{hi}) can direct their migration into injured tissues during inflammation [189-191]. Similarly, there are two major monocyte subsets that exist in human circulation, CD14^{hi}CD16⁻ and CD14^{lo}CD16⁺ monocytes that display similar homing potential as their murine counterpart [189, 190, 192]. Despite extensive amount of research that has been done to characterize monocyte chemokine receptor expression and monocyte trafficking, not as much is known about chemokine receptor expression pattern once monocyte reach the target tissue and mature into macrophages. In addition, it is also likely that macrophage chemokine receptor expression pattern, and thus, macrophage motility can be affected by their polarization states.

Distinct macrophage polarization states are associated with unique macrophage chemokine secretion pattern [10]. Classically activated M1 macrophages have been reported to secrete many proinflammatory chemokines that actively recruit leukocytes including proinflammatory monocytes, neutrophils and activated T cells into the inflammation site [10, 182]. Activation of the TLR signaling pathway results in the transcription of NF- κ B dependant chemokines, including CXC chemokine ligand (CXCL) 1,2,3,5,9, and 10, CC chemokine ligand (CCL) 11, 17 and 22, and IL-8,

monocyte chemoattractant protein-1 (MCP-1), macrophage inflammatory protein-1 α (MIP-1 α) MIP-1 β , regulated on activation, normal T cell expressed and secreted (RANTES) [10, 13]. On the contrary, activation of the M2a and M2c polarization states results in the inhibition of proinflammatory chemokines secretion. IL-4 and IL10 have been reported to inhibit the production of CXCL9, CXCL10 and RANTES through the inhibition of NF- κ B pathway [1, 10, 193]. In addition to inhibiting proinflammatory chemokines generation, M2 macrophages can also secrete chemokines that recruit immune cells involved in Th2 responses, tissue repair and remodeling and immunoregulation [10]. M2a macrophages produce CCL17, 18, 22 and 24, which can recruit basophils, eosinophils, and Th2 cells and regulatory T cells (Treg) [10, 194, 195]. M2b macrophages have been reported to produce CCL1, which can recruit eosinophils, Th2 cells and T reg [10]. Lastly, M2c macrophages can produce CCL16, CCL18 and CXCL13, which can recruit eosinophils [10]. Overall, chemokines produced by M2 macrophages promote the recruitment of leukocytes that display anti-inflammatory effects compared to the proinflammatory nature of chemokines produced by M1 macrophages.

Compared to monocytes, the chemokine receptor expression pattern on macrophages is not as understood. In addition, given the diversity and complexity of macrophage polarization states, chemokine receptor expression patterns on these macrophages are likely to be just as complex. M2a macrophages have been reported to express CXC receptor (CXCR) type 1 and CXCR2 that are functionally active [10]. M2c macrophages have been reported to display higher expression level of both CCR2 and CCR5, which are proinflammatory chemokine receptors [10, 196]. However, these

macrophages do not migrate toward the corresponding ligands. In fact, it was reported that CCR2 and CCR5 expression on M2c macrophages serve to act as decoy receptors that function to scavenge the proinflammatory ligands from the environment [10, 197]. Some earlier reports have also reported CCR7 as a marker for pro resolution macrophages that participates in macrophage egress from the atherosclerotic plaque to the nearby draining lymphatics [198, 199]. However, further research has shown that CCR7 may not be involved in this process [41]. In fact, CCR7 gene expression has been demonstrated to be upregulated in classically activated M1 macrophages [129, 200]. Overall, not much is known regarding the chemokine receptor expression pattern on macrophages and how it relates to their polarization states.

2.3.4 Macrophages in chronic inflammatory disease: atherosclerosis

Macrophages are central in the execution of host's immune response. However, as mentioned previously, defects in macrophage biochemical functions including the regulation of macrophage polarization, efferocytosis and migration responses can result in the generation of macrophages that are pathogenic. Atherosclerosis is a chronic vascular disease that is characterized by continuous recruitment of proinflammatory monocytes into the lesion site [7, 16, 17, 167, 201]. This can result in the accumulation of activated macrophages in the vascular wall, which contributes to the maintenance of a high level of proinflammatory mediators like proinflammatory cytokines, reactive oxygen species and proteases in the global environment. Recent study performed by Khallou-Laschet et al has indicated the presence of a progressive shift in macrophage polarization during atherosclerosis progression, with M2 macrophages dominating early lesion and M1 macrophages dominating late, advanced lesion [202]. In addition, the combination of

high leukocyte presence, which eventually turns apoptotic, and defective efferocytosis, eventually results in the generation of necrotic core within the vascular wall [7, 167]. Overall, this combines to create an inflammatory environment with a massive M1/M2 macrophage imbalance. Another recent publication by van Gils et al also highlighted the importance of macrophage motility in atherosclerosis [203]. It was found that netrin-1 secretion in atherosclerotic plaque could block macrophage migration toward select chemokines which then resulted macrophage retention in the vascular wall, leading to atherosclerosis progression [203]. Conversely, deletion of netrin-1 was associated with increased macrophage egress and reduced plaque burden [203]. Overall, these studies suggest that macrophages can have a major impact on inflammation outcome. Given the recent understanding of the active nature of resolution process, more effort is being invested into understanding where the pro resolution mechanisms fail in chronic disease like atherosclerosis and what can be done to reverse chronic inflammation.

Recently, our group has discovered a unique population of macrophages that express Sdc-1 in murine AAA disease model [26]. Macrophage Sdc-1, which has been described to participate in the regulation of cell adhesion and motility, was found to be protective in this model. This agrees with other reports, including studies conducted in murine DTH and myocardial infarction, where Sdc-1 expression has been found to be protective [27, 28]. Thus, Sdc-1 expression can potentially be important in the regulation of macrophage functions and thus affects the inflammation outcome.

2.4 Syndecan-1 in inflammation

Syndecans are a family of type I transmembrane heparan sulfate and chondroitin sulfate proteoglycans that consist of 4 family members, Sdc-1, -2, -3 and -4 [18-20].

Syndecans consist of an extracellular domain that is decorated with sulfated sugar chains, a short transmembrane domain, and an intracellular domain that is highly conserved across the different members and across different species. Syndecans are known to be expressed on various cell types throughout many different stages of developments. While Sdc-1 is normally expressed on endothelial cells and plasma cells surface in adult tissues, its expression on other cell types can be induced by soluble growth factors, such as TGF- β , that are released at the site of injury [22-25, 79]. Sdc-2 can be found on mesenchymal cells and endothelial cells, while Sdc-3 can be found mostly on neural cells [19, 20]. Lastly, Sdc-4 is expressed on most cell types, though at a lower level than other syndecans [19, 20].

Through its heparan sulfate sugar chain, syndecans can bind and regulate the activity of many soluble and insoluble ligands, including extracellular matrix (ECM) components (e.g. fibronectin, collagen), growth factors (e.g. fibroblast growth factor (FGF), TGF- β , platelet derived growth factor (PDGF)), proteases (e.g. MMP-1,2,9, cathepsin G, neutrophil elastase), and cytokines (eg. IL-8) and chemokines (eg. IFN- γ , RANTES, MIP-1 α , MIP-1 β) [18, 204]. Syndecans can influence these ligands' biological functions by affecting their availability, stability and compartmentalization [18, 205]. Thus, through its role as a cell surface co-receptor, syndecans are able to regulate events relevant to tissue repair and chronic injury responses, which include cell migration and proliferations, cell-substrate interactions, wound healing and matrix remodeling.

Within the syndecan family, Sdc-1 is emerging to be an important regulator of inflammatory response. Several studies utilizing Sdc-1^{-/-} animals have described the

potential role of Sdc-1 in regulating leukocyte recruitment during inflammation [28, 206, 207]. In a murine model of DTH, which is commonly used to study cell-mediated immune responses, absence of Sdc-1 expression was associated with prolonged inflammation, increased infiltration of leukocytes, and increased production of proinflammatory cytokines, including TNF- α , Il-6 and RANTES [28]. Similarly, in a murine model of nephritis, increased leukocyte adhesion and recruitment, increased proinflammatory cytokines production and impaired wound healing response were observed in the absence of Sdc-1 [206]. Studies in animal model of toxic shock also demonstrated similar outcomes [207].

Sdc-1 has also been described to affect the wound healing responses in several inflammatory disease models. In a myocardial infarction model, mice lacking Sdc-1 expression demonstrated abnormal tissue healing, characterized by fragmented collagen fibers, which was attributed to higher MMP proteolytic activity in the tissue [27]. On the other hand, increased Sdc-1 expression was reported to attenuate the inflammatory response, improve tissue healing and protect against cardiac dysfunction [27, 208]. Recent publication from our research group also demonstrated that the absence of Sdc-1 resulted in the exacerbation of AAA progression [26]. Sdc-1 absence was resulted in increased elastin degradation and increased MMP proteolytic activity in the vascular wall. Increased leukocyte infiltration into the vascular wall was also observed. Overall, these studies suggest that Sdc-1 expression during inflammation can result in dampened inflammatory responses.

Of particular interest is the expression of Sdc-1 on macrophages. Activation of the cAMP-PKA signaling pathway, which is one of the cellular signaling pathways that

participates in the resolution of inflammation, has been shown to regulate macrophage Sdc-1 expression [29, 209]. The work performed by Kim et al has also demonstrated the involvement of this particular signaling pathway in the regulation of macrophage migration response in anthrax, as mentioned previously [29]. Indeed, Sdc-1 was found to be one of the downstream targets activated via the cAMP-PKA pathway in macrophages following their exposure to the anthrax toxin. It was also confirmed that macrophage migration response following anthrax toxin exposure was significantly impaired in the absence Sdc-1 expression. Given the importance of macrophage motility in ensuring successful inflammation resolution, Sdc-1 can potentially be an important factor in the regulation of inflammation.

2.5 Summary

The extent to which Sdc-1 affects macrophage polarization and functions has not been well defined. Recent findings have brought into light the active nature of the resolution of inflammatory response, which involves both emigration and efferocytosis of monocyte derived cells out of the site of inflammation through nearby lymphatic vessels. Both of these processes may be impaired in chronic inflammatory diseases, which are usually associated with accumulation of macrophages at the disease site. While the mechanisms for impaired efferocytosis and emigration remain to be determined, the failure of macrophages to switch to a phenotype that express Sdc-1 may contribute to impaired motility and phagocytic function, which subsequently contributes to the maintenance of chronic inflammatory state. Thus, it is important to understand how Sdc-1 expression influences the clearance of monocyte derived cells out of the inflamed vascular wall, promoting resolution. This can also offer new insight in the regulation of

inflammation and lead to the discovery of pathways that can be utilized to promote its resolution.

CHAPTER 3

SYNDECAN-1 EXPRESSION ON MACROPHAGES IS PROTECTIVE IN ATHEROSCLEROSIS AND IS ASSOCIATED WITH M2 POLARIZATION STATE AND ENHANCED MOTILITY

Sdc-1 expression has been reported to be protective in many inflammatory diseases. Recently our group has reported that macrophage Sdc-1 expression is upregulated and is protective during the progression of AAA. Despite these reports, the mechanisms by which Sdc-1 promotes the polarization of monocyte derived cells and their functions have not been well defined. Given the important role that macrophages play during the course of inflammatory response and their abundance in chronic inflammatory diseases, we seek to define the mechanisms by which Sdc-1 expression can alter macrophage functions and affect inflammation outcome. The effects of macrophage Sdc-1 expression on inflammation outcome will also be examined in a murine model of atherosclerosis

3.1 Introduction

Atherosclerosis is a chronic cardiovascular disease that serves as the number one killer in developed countries and is quickly becoming the major cause of deaths in many developing countries [201, 210, 211]. In the United States alone it accounts for more than 1 million deaths, while worldwide, it is responsible for more than 19 million deaths annually [210, 211]. Atherosclerosis progresses over the course of many years and in many cases, is asymptomatic during its early stages. It is characterized by progressive

thickening and narrowing of the blood vessel, which results in the gradual blocking of the arteries, compromising blood flow in the process. Consequently, atherosclerosis can serve as the underlying cause of many cardiovascular diseases, including myocardial infarction, strokes and peripheral artery disease [210, 212].

Unlike acute cases of inflammation, where leukocytes ultimately leave the site of inflammation, atherosclerosis is characterized by the persistence of activated macrophages that fail to leave the atherosclerotic plaque. Key processes necessary for the resolution of inflammation, including decreased influx of inflammatory cells efferocytosis, and macrophage egress from the inflammation site have all been shown to be defective in advanced atherosclerotic lesions [7, 167]. This can result in the accumulation of apoptotic cells in the plaque, secondary necrosis, necrotic core generation and recruitment of additional monocytes that mature into proinflammatory macrophages in the lesion. [7, 167]. At the same time, the clearance of inflammatory macrophages and the generation of anti-inflammatory cytokines associated with the efferocytosis process are also diminished. Overall, these result in the maintenance of a proinflammatory environment and the generation of a pro-atherosclerotic inflammatory feedback loop that stimulates plaque progression.

Macrophages often represent the dominant leukocyte population in chronic inflammation; in atherosclerosis both early and late lesion areas consist of macrophage rich regions [7, 8]. Microenvironmentally derived signals can give rise to distinct macrophage subsets that are polarized with specific functional properties, including classically activated, proinflammatory M1 macrophages, and alternatively activated M2 macrophages [10, 13, 14, 129, 132, 140]. M2 macrophages can be further categorized

into at least 3 individual subtypes functioning in allergy and parasite defense (M2a), immunoregulation (M2b), or tissue repair and remodeling (M2c) [10, 13, 14, 130].

Recent studies indicate that distinct macrophage subsets are present in mouse models of atherosclerosis; early lesions have been associated with M2 macrophages, while more advanced atherosclerosis is associated with an M1 phenotype [202]. Indeed, this suggests that lesion progression may correlate with M1:M2 ratio and the balance between these different subsets may affect the dynamic and outcome of the disease. While regression of atherosclerosis lesion is not commonly found in human cases, studies using murine models of atherosclerosis have indicated that regression can be achieved under certain conditions. Robust lipoprotein profile change, achieved through transplantation of atherosclerotic arterial segment from Apolipoprotein E (ApoE)^{-/-} mice to wild type (WT) recipients has been shown to result in rapid atherosclerosis regression, which was explained by emigration of foam cells from the lesion to the lymph nodes [198]. More recent study using an ApoE complementation model also indicated that plaque regression could be linked to suppressed monocytes recruitment into the atherosclerotic plaque [41].

A recent report from van Gils et al described the expression of the negative guidance cue netrin-1 in foam cells; netrin-1 was found to promote macrophage retention through inhibition of cytoskeletal reorganization, effectively blocking directed migration [203].

Notably, targeted deletion of netrin-1 improved macrophage emigration and atherosclerotic outcomes. Overall, these findings reveal that dampening inflammatory influx and restoring macrophage motility potential in the lesion may facilitate macrophage egress and plaque regression

Syndecan-1 (Sdc-1) is a member of a family of cell surface proteoglycans that has been reported to participate in the regulation of events relevant to tissue repair and chronic injury responses, including cell-substrate interactions, matrix remodeling and cell migration and proliferation [19-21, 204]. Sdc-1 expression, which can be highly regulated by soluble growth factors that are released at the sites of injury, has been reported to be protective in various inflammatory models, including delayed type hypersensitivity, nephritis, toxic shock, myocardial infarction and abdominal aortic aneurysm (AAA) [26, 28, 206-208]. Expression of Sdc-1 on macrophages, which we have previously demonstrated to be protective in the murine AAA model, is of particular interest, given the important role that macrophages play in atherosclerosis inflammation [26]. Of note, induction of macrophage Sdc-1 expression is controlled through the cAMP/ protein kinase A signaling pathway, which has been shown to be anti-inflammatory [29, 209].

In this chapter, we aim to define the functional role of Sdc-1 in atherosclerosis. Using a murine atherosclerosis model, we have identified the presence of a macrophage subset in the atherosclerotic wall that expressed Sdc-1. Deficiency in Sdc-1 expression was found to exacerbate wall inflammation, which resulted in increased plaque burden. In vitro characterization of murine Sdc-1⁺ macrophages, which can be generated through the activation of cAMP/protein kinase A signaling pathway or through exposure to adenosine, indicates that Sdc-1 expression defines an anti-inflammatory population with high motility. We also found that the absence of Sdc-1 was associated with defective actin reorganization, which resulted in dampened macrophage migration. Analysis of human macrophages confirmed Sdc-1 expression in an alternatively activated, motile

population. In addition, we identified Sdc-1 positive macrophages in human atherosclerotic plaque. Collectively, these results reinforce the physiological significance of macrophage motility as an endogenous modulator of inflammatory response and further suggest Sdc-1 as a crucial component in the regulation of macrophage motility response.

3.2 Results

3.2.1 Macrophage associated Sdc-1 expression attenuates atherosclerotic lesion formation

Administration of high fat Western diet to ApoE^{-/-} mice has been demonstrated to significantly accelerate atherosclerotic plaque formation. To evaluate the involvement of macrophage Sdc-1 expression during atherosclerosis progression, ApoE^{-/-} mice were maintained on high fat Western diet for 8 weeks. Immunohistochemistry was then utilized to characterize Sdc-1 expression in concert with macrophage infiltrate within the atherosclerotic plaque obtained from the animals. We found that Sdc-1 expression was upregulated within the atherosclerotic plaque, with most Sdc-1 positive cells localized to the intima and adventitia, while little to no expression of Sdc-1 was detectable in the native aorta (Fig.3.1A). In addition, we observed an association between macrophage and Sdc-1 positive staining, which was confirmed by double immunofluorescence staining. As illustrated, Sdc-1 expression was found to colocalize with Mac-3 positive cells, consistent with the notion that Sdc-1 expression was specific to infiltrating macrophages (Fig.3.1B). To evaluate the involvement of Sdc-1 in atherosclerosis progression, ApoE^{-/-}Sdc-1^{-/-} mice were enrolled in the study and immunohistochemistry was then utilized to characterize macrophage infiltration within the plaque. Sdc-1

deficient mice have been previously characterized as healthy, with normal growth, reproduction, tissue morphology, hematologic profile and serum chemistry parameters [207, 213]. We observed an increase in the atherosclerotic lesion size in the absence of Sdc-1 expression, which was confirmed by measurement of atherosclerotic lesion area in both ApoE^{-/-} and ApoE^{-/-}Sdc-1^{-/-} mice (Fig.3.1C). Overall, these results suggest that deficiency in Sdc-1 expression can exacerbate vascular wall inflammation, which results in increased atherosclerotic lesion formation.

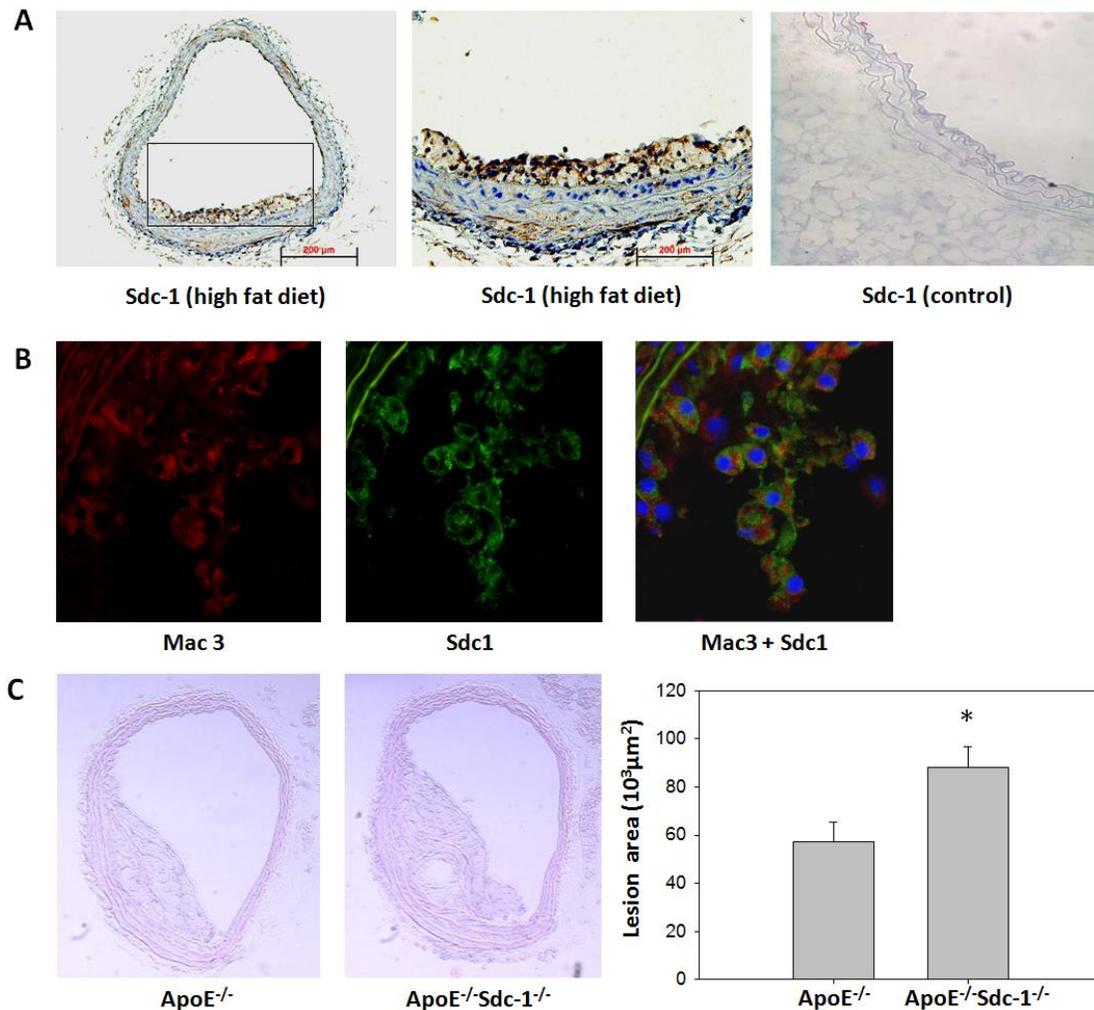


Figure 3.1. Macrophage expressed Sdc-1 attenuates experimental atherosclerosis lesion formation. **A.** Immunohistochemistry staining of Sdc-1 within the atherosclerotic plaque following 8 weeks of high fat diet and in control aorta. **B.** Representative images showing immunofluorescence staining of macrophages (Mac3, Alexa 594, red), Sdc-1 (fluorescein, green) and 4',6-diamidino-2-phenylindole nuclear stain (DAPI, blue) within the atherosclerotic plaque. **C.** Representative images showing the atherosclerotic lesion size in ApoE^{-/-} and ApoE^{-/-}Sdc-1^{-/-} mice following 8 weeks of high fat diet. The extent of lesion formation was reported as lesion area (n=19). *P<0.05. All data represent the mean ± standard error of the mean (SEM).

3.2.2 Sdc-1 is expressed on macrophages displaying M2 characteristics

Given the observed importance of macrophage expressed Sdc-1 to the localized inflammatory response, we sought to define the functional polarization state of macrophages expressing Sdc-1. Initial studies to characterize the polarization of Sdc-1⁺ macrophages were performed utilizing flow cytometry to define the cell surface expression of MHCII, CD86 and PD-L2; both MHCII, an antigen presenting molecule, and CD86, a co-activating molecule, have been reported to be upregulated on M1 and M2b macrophages [10], while PD-L2, a Th2 stimulated molecule that inhibits T cell proliferation, has been reported to be highly upregulated on M2a macrophages and to a lower extent, on M1 macrophages [156]. While the presence of M1 and M2 like macrophages in atherosclerotic plaque has been previously demonstrated, the involvement of immune complexes, necessary for the generation of M2b macrophages, in atherosclerosis progression has not been widely reported [202, 214]. Thus, we opted to characterize MHCII, CD86 and PD-L2 surface expression on Sdc-1 enriched macrophages and polarized standard populations, which include: (i) classically activated M1 (Ifn- γ and LPS treated), (ii) alternatively activated M2a (IL-4 treated), and (iii) regulatory M2c (IL-10 treated). Biochemical and functional activities, such as generation of nitrite, arginase activity, generation of IL-10 and phagocytosis activity were also characterized. Macrophage Sdc-1 induction in vitro was achieved using cAMP or adenosine treatment (Fig.3.2A). While macrophage Sdc-1 expression in response to cAMP-PKA signaling pathway activation has been shown previously [209], macrophage Sdc-1 expression in response to adenosine, an endogenous anti-inflammatory agent that exerts anti-inflammatory effects on macrophages, has not been well characterized. Flow

cytometry analysis of control macrophage populations showed that Sdc-1 was not expressed on M1, M2a or M2c macrophages (Fig.3.2B). While we have identified 2 in vitro conditions to induce Sdc-1 expression, subsequent subtype characterizations was performed using adenosine induced Sdc-1 expression, unless noted otherwise. As expected, M1 polarized macrophages displayed characteristically high expression of MHCII, and CD86 (Fig.3.2C). M2a polarized macrophages were characterized as CD86⁺ with high expression of PD-L2 (Fig.3.2C). However, M2c macrophage does not positively correlate with MHCII, CD86 or PD-L2 (Fig.3.2C), consistent with reports that IL-10 deactivates macrophages [156]. Sdc-1 expressing macrophages, which can be enriched after treatment with a cAMP or adenosine, displayed strongest similarity to M2c macrophages with a MHCII^{low}, CD86^{low} and PD-L2⁻ signature (Fig.3.2C).

Macrophage populations were further characterized for soluble mediators. We found elevated nitrite level only in M1 population (Fig.3.2D), while arginase activity was enhanced only in M2a population (Fig.3.2E). We observed a significant increase in IL-10 secretion in our Sdc-1⁺ population, though not as high as M2c macrophages (Fig.3.2F). Finally, we characterized phagocytosis, which has been reported to be upregulated in M2 macrophages [163, 215, 216], and found that activity was significantly elevated in all populations compared to M1 (Fig.3.2G).

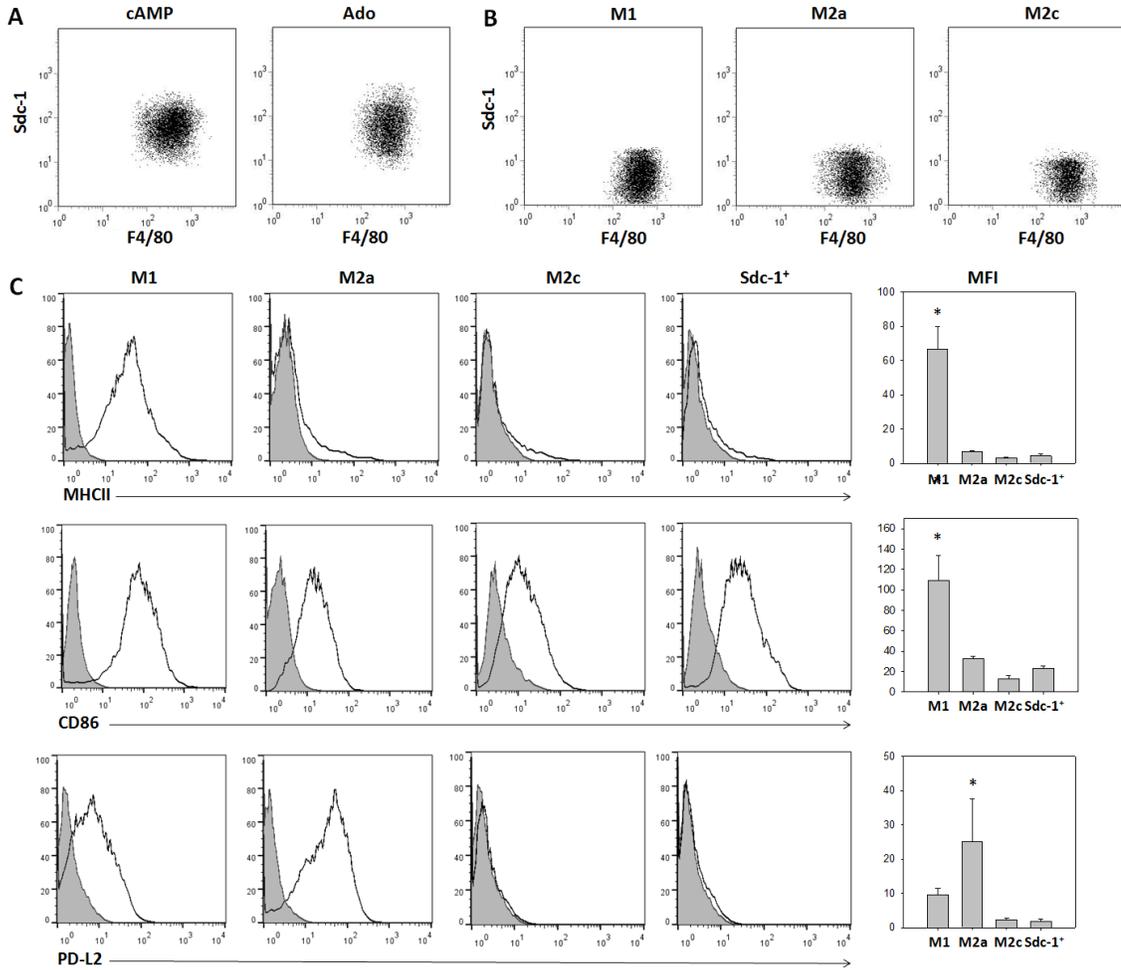


Figure 3.2A-C. Macrophage Sdc-1 expression is associated with alternatively activated M2 polarization state. **A.** Sdc-1 expression was analyzed on murine peritoneal macrophages following exposure to cAMP (100 μ M) or adenosine (Ado, 375 μ M). **B.** Sdc-1 expression was measured on M1 (IFN- γ , 100U/mL + LPS, 100ng/mL), M2a (IL-4, 20ng/mL), and M2c (IL-10, 100ng/mL) polarized standard population. **C.** Sdc-1 expressing macrophages were examined against standard populations for surface expression of M1 (MHCII, CD86) and M2a (PD-L2) markers. Expression level was quantified as mean fluorescence index (MFI) (filled: IgG control, black line: experimental; n=3-4). *P<0.05. All data represent the mean \pm SEM.

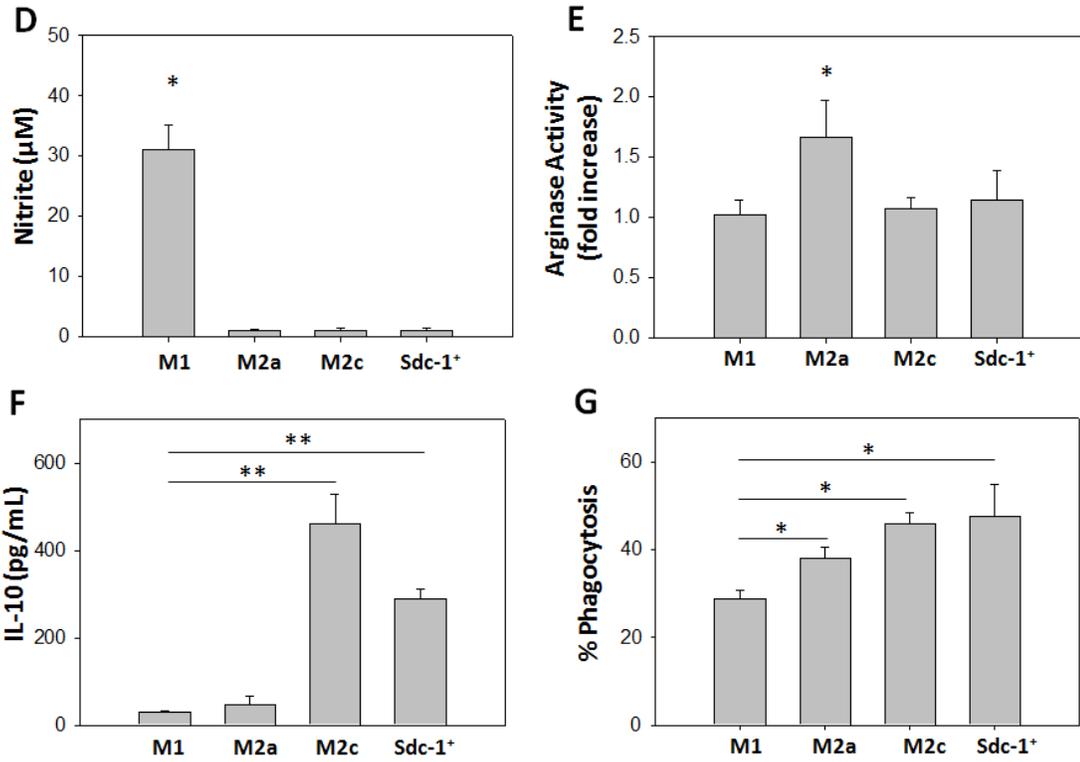


Figure 3.2D-G. Sdc-1 expression is associated with alternatively activated M2 polarization state. **D.** iNOS activity was characterized via Griess reaction using conditioned media of M1, M2a, M2c and Sdc-1⁺ macrophages (n=3). **P<0.01. **E.** Arginase activity was measured from conditioned media of polarized macrophages and measured as fold increase compared to unstimulated peritoneal macrophages (n=4-5). *P<0.05. **F.** IL-10 was measured from the conditioned media of immunostimulated M1, M2a, M2c and Sdc-1⁺ macrophages using ELISA (n=3). **P<0.01. **G.** Phagocytosis of M2a, M2c and Sdc-1⁺ macrophages was compared against M1 macrophages. % phagocytosis was quantified through fluorescent bead uptake and analyzed using FACS (n=3). *P<0.05. All data represent the mean ± SEM.

3.2.3 Sdc-1⁺ macrophages display low leukocyte chemo-attractive potential compared to M1 macrophages

Although it is a challenge to effectively categorize Sdc-1 expressing macrophages into any previously defined M2 subtype, it is clear that Sdc-1 expression is not consistent with a classically activated M1 phenotype and subsequent quantitative real-time reverse transcription polymerase chain reaction (PCR) confirmed this distinction. As expected, higher level of proinflammatory genes and chemokines were expressed in M1 compared to Sdc-1 enriched macrophages (Table 3.1 and 3.2). Given the impact that leukocytes recruitment can have on inflammation outcome, we examined the chemo-attractive potential of conditioned media generated from M1 or Sdc-1 enriched populations toward purified circulating monocytes or activated CD4 T cells. Circulating monocytes, which mature into macrophages in the tissue, and activated CD4 T cells have both been demonstrated to be actively recruited into the atherosclerosis plaque [41, 217]. As expected, we observed significantly reduced migration response from both monocytes and T cells to conditioned media obtained from Sdc-1⁺ macrophages compared to condition media obtained from M1 macrophages (Fig.3.3). This result suggest that while M1 macrophages can contribute to the progression of inflammation through its recruitment of inflammatory leukocytes, Sdc-1⁺ macrophages can dampen inflammatory response by limiting leukocyte recruitment into the inflammation site. This finding also suggests that macrophage Sdc-1 expression should correlate with reduced inflammatory response in vivo.

Table 3.1 mRNA expression profile of inflammatory genes in M1 compared to Sdc-1⁺ macrophages. RNA was extracted from M1 or MACS enriched Sdc-1⁺ macrophages and analyzed on the chemokines and receptors RT1 profiler PCR array; expression were normalized to GAPDH and were reported as expression index. All data represent the mean (n=3).

Gene	M1:Sdc-1 ⁺	Gene Information
GM-CSF	29	Granulocyte differentiation factor
IL1 α	14	Proinflammatory cytokine
NF κ β	6	Transcription factor for many inflammatory genes
MyD88	5	Adapter protein that participates in the activation of NF κ β
TNF α	103	Proinflammatory cytokine

Table 3.2 mRNA expression profile of chemokines in M1 compared to Sdc-1⁺ macrophages. RNA was extracted from M1 or MACS enriched Sdc-1⁺ macrophages and analyzed on the chemokines and receptors RT1 profiler PCR array; expression were normalized to GAPDH and were reported as expression index. All data represent the mean (n=3).

Gene	M1:Sdc-1 ⁺	Gene Information
CCL2/MCP1	78	Monocyte chemoattractant
CCL4/MIP1 β	60	Induce neutrophil superoxide production
CCL5/RANTES	354	TNF α and IL1 β inducible, T cell recruitment
CCL7/MCP-3	100	Monocyte recruitment
CCL8/MCP-2	27	Monocyte, T cell and NK cell recruitment
CCL12/MCP-5	834	Monocyte recruitment
CXCL1	32	Neutrophil and monocyte recruitment
CXCL2	67	Neutrophil recruitment
CXCL9	99,768	IFN- γ inducible, T cell and monocyte recruitment
CXCL10	5,019	IFN- γ inducible, T cell and NK cell recruitment
CXCL11	211	IFN- γ inducible, T cell recruitment

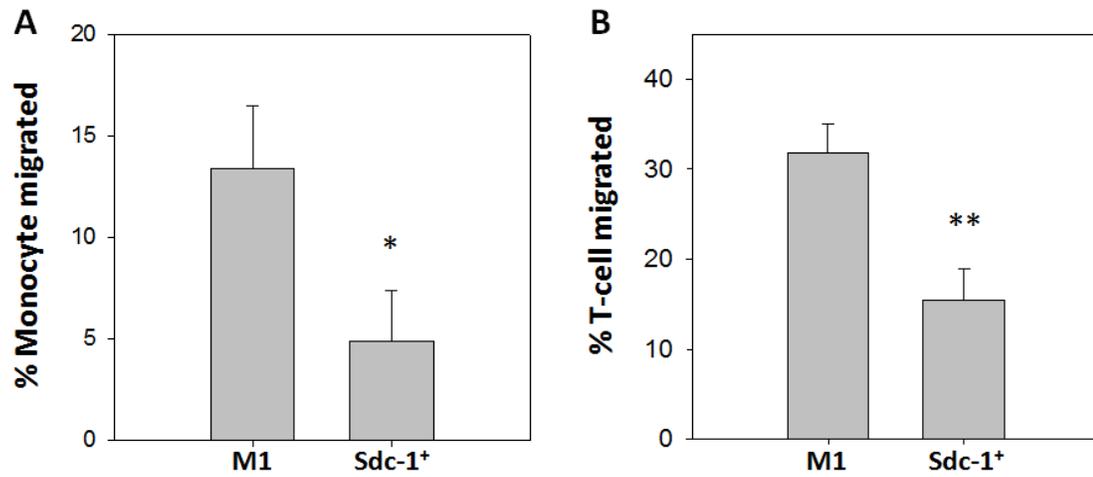


Figure 3.3. Sdc-1 expression is associated with reduced leukocyte chemoattractive potential. Leukocyte chemoattractive potential was measured by transwell chemotaxis of **A.** murine circulating monocytes and **B.** murine activated CD4 T cells to conditioned media from M1 or Sdc-1⁺ macrophages. Cells that migrated to the lower chamber were counted and reported as % migrated (n=3). *P<0.05, **P<0.01. All data represent the mean \pm SEM.

3.2.4 Macrophage Sdc-1 expression is associated with reduced plaque inflammation

Our study utilizing murine atherosclerotic model indicates that Sdc-1 plays a protective role in atherosclerosis progression, which is supported by our in vitro characterizations of Sdc-1⁺ macrophages. To evaluate the effect of Sdc-1 expression on the global inflammatory environment within the vascular wall, immunohistochemistry was utilized to characterize the expression of iNOS within the atherosclerotic lesion of both ApoE^{-/-} and ApoE^{-/-} Sdc-1^{-/-} mice. As mentioned previously, we observed discrete regions of Sdc-1 expression, which was co localized with macrophages in the intima and adventitia region. In contrast, we observed a different iNOS expression pattern within the plaque which indicated that distinctiveness of the Sdc-1⁺ macrophages from the M1 polarized macrophages. In addition, increased iNOS presence within the vascular wall of ApoE^{-/-} Sdc-1^{-/-} mice suggested an exaggerated inflammatory response in the absence of Sdc-1 expression (Fig.3.4).

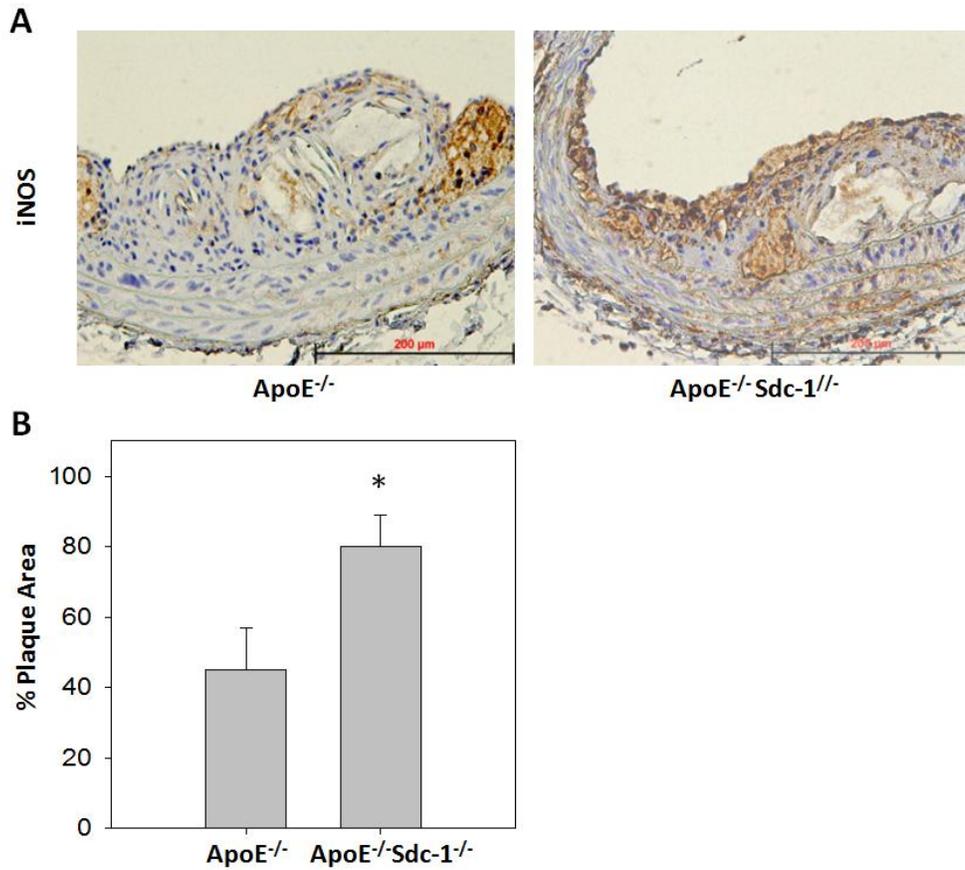


Figure 3.4. The absence of Sdc-1 expression is associated with increased inflammatory response. **A.** Immunohistochemistry staining of iNOS within the atherosclerotic plaque of ApoE^{-/-} and ApoE^{-/-}Sdc-1^{-/-} mice following 8 weeks of high fat diet. **B.** The extent of iNOS expression was reported as % of plaque area (n=3). *P<0.05. All data represent the mean ± SEM.

3.2.5 Each macrophage polarization state is associated with distinct motility and adhesion response, which is both reduced in the absence of Sdc-1 expression

While our findings support the hypothesis that Sdc-1 expression on macrophages is indicative of a M2 polarization state, the mechanisms by which this protective effect can be achieved in vivo is unknown. Given that Sdc-1 has been traditionally studied in the context of cell adhesion and migration, it is likely that Sdc-1 expression can affect macrophage motility response. Macrophage intrinsic motility was first investigated by looking at the migration of M1, M2a, M2c and Sdc-1⁺ macrophages toward fetal bovine serum (FBS) using a transwell chemotaxis experimental setup. To investigate the effect of macrophage Sdc-1 expression on motility, the experiment was also performed using Sdc-1^{-/-} macrophages. M1 macrophages displayed significantly attenuated migration response toward FBS compared to M2 and Sdc-1⁺ macrophages (Fig.3.5A). In addition, motility was also found to be significantly reduced in the absence of Sdc-1 expression (Fig.3.5.B). We next investigated whether macrophage adhesion was related to the altered intrinsic motility associated with different polarization states and Sdc-1 expression by looking at the adhesion of M1, Sdc-1⁺ and Sdc-1^{-/-} macrophages to fibronectin coated surface. Result from this experiment suggested that reduced intrinsic motility in macrophages was associated with increased adherence. M1 and Sdc-1^{-/-} macrophages both displayed significantly stronger adhesion compared to macrophages expressing Sdc-1 (Fig.3.5C). The regulation of cell motility and cell adhesion is closely associated with cytoskeletal activation. The actin cytoskeleton has been reported to be important in the regulation of macrophage migration response [218]. Consistent with the reduced intrinsic motility, absence of Sdc-1 was associated with reduced actin activation

in response to adenosine exposure (Fig.3.5.D). These findings suggest that macrophage motility is potentially an additional mechanism by which macrophages orchestrate the inflammatory response. In addition, Sdc-1 expression on macrophages can serve as an important modulator in influencing inflammation outcome.

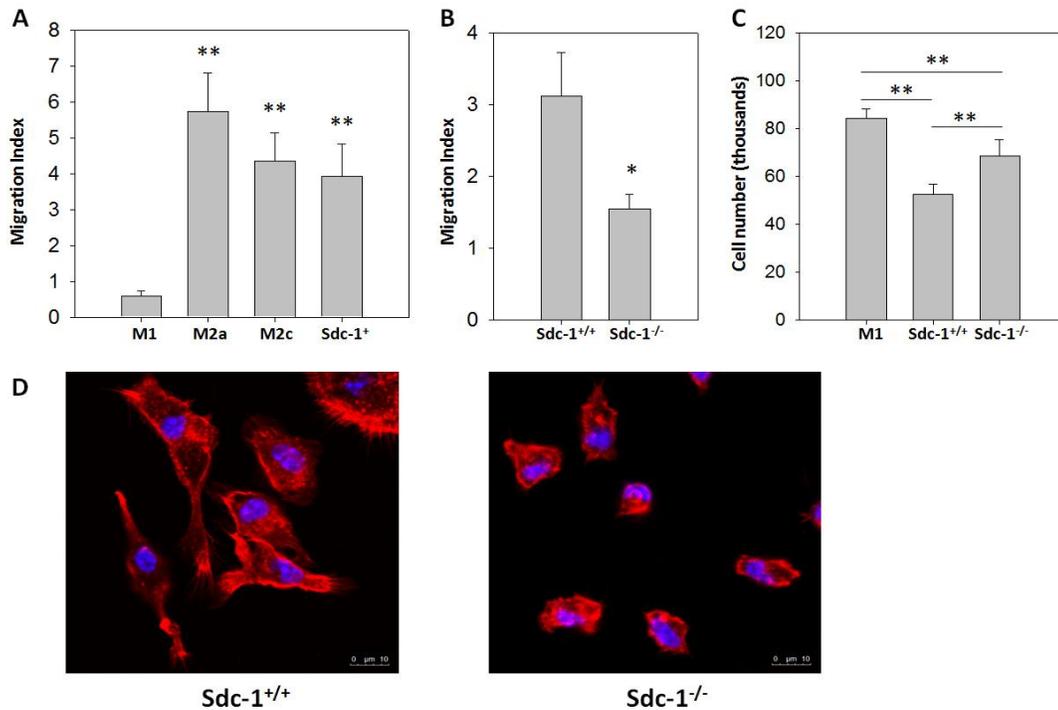


Figure 3.5. Polarized macrophages display distinct migration and adhesion. **A-B** Intrinsic motility was analyzed using transwell migration to FBS. Polarized macrophages were placed into the upper chamber of a transwell and macrophages that migrated to the lower surface of the membrane was stained, counted and reported as migration index. **A.** Intrinsic motility of standard populations and Sdc-1⁺ macrophages (n=4). **P<0.01. **B.** Intrinsic motility of Sdc-1^{+/+} and Sdc-1^{-/-} macrophages (n=5). *P<0.05. **C.** Macrophage adhesion was characterized using fluorescently labeled M1, Sdc-1^{+/+} and Sdc-1^{-/-} macrophages that was allowed to adhere onto fibronectin coated surface (n=3-4). **P<0.01. **D.** Sdc-1^{+/+} and Sdc-1^{-/-} macrophages were treated with adenosine and stained for actin (red) and nuclei (blue). All data represent the mean \pm SEM.

3.2.6 Efferocytosis as a regulator of macrophage Sdc-1 expression

Our characterizations of Sdc-1⁺ macrophages have suggested that Sdc-1 expression is associated with both anti-inflammatory polarization states and increased intrinsic motility. While expression of Sdc-1 on macrophages can be obtained in vitro through exposure to cAMP or adenosine, the point of control for Sdc-1 expression in vivo is still unknown. Efferocytosis, a process that involves the clearance of apoptotic cells from the site of inflammation, has often been suggested as a physiological proresolving signal that can influence macrophage polarization [15, 16, 167]. Efferocytosis has also been suggested to result in macrophage egress from inflammatory sites [49], a process that is presumably associated with enhanced motility. Moreover, efferocytosis has been reported to result in macrophage adenosine production [219]. Overall, these findings suggest that efferocytosis can potentially be involved in the regulation of macrophage Sdc-1 expression during inflammation. Indeed, flow cytometry analysis demonstrated that macrophage exposure to apoptotic cells resulted in the upregulation of macrophage Sdc-1 expression (Fig.3.6), suggesting a physiologic program for expression that is consistent with inflammatory resolution.

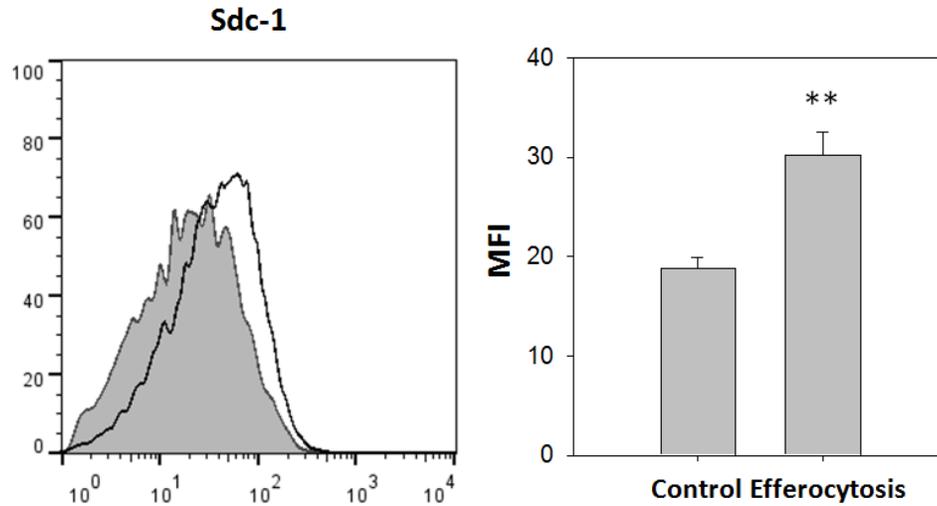


Figure 3.6. Efferocytosis induces macrophage Sdc-1 expression. Sdc-1 expression was analyzed using flow cytometry following macrophage exposure to apoptotic cells and was quantified as MFI (filled: control, black line: efferocytosis; n=5). ** $P < 0.01$. All data represent the mean \pm SEM.

3.2.7 Sdc-1 expression on human macrophages is associated with M2 polarization and increased motility.

Thus far our results have demonstrated that Sdc-1⁺ macrophages display features characteristic of alternatively activated M2 polarization state. To verify Sdc-1 expression and to correlate our findings in murine macrophages with human macrophages, we decided to characterize Sdc-1 expression on human carotid plaque and polarized THP1 derived human macrophages. Indeed, Sdc-1 was found to be present in the human carotid plaque and similar to murine atherosclerotic lesion, its expression was found in the vicinity of macrophages (Fig.3.7A). For our in vitro characterization, M1 polarization on human macrophages was achieved using IFN- γ and LPS treatment, while Sdc-1 expression was triggered by cAMP treatment. Surface expression of human leukocyte antigen-D related (HLA-DR), an MHC class II surface receptor and a marker for M1 polarization [220] and Sdc-1 were analyzed by flow cytometry. As expected, flow cytometry analysis indicated high expression level of HLA-DR on M1 macrophages but not on cAMP treated macrophages (Fig.3.8A). On the contrary, M1 macrophages were found to display no Sdc-1 on their surface, while cAMP treated macrophages were found to upregulate their Sdc-1 expression (Fig.3.8B). Similar to the results obtained using murine macrophages, macrophage Sdc-1 expression was associated with enhanced motility, while M1 macrophages displayed significantly dampened motility response (Fig.3.8C). Overall, our findings suggest that Sdc-1 expression on both human and murine macrophages is associated with M2 polarization states and enhanced macrophage motility.

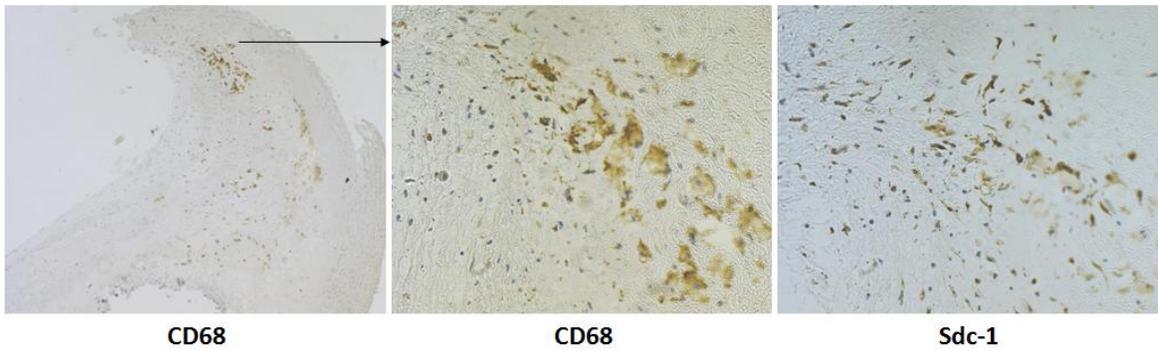


Figure 3.7. Sdc-1 is expressed on macrophages in human carotid plaque. Representative immunohistochemical staining of macrophages (CD68) and Sdc-1 in human carotid plaque sections.

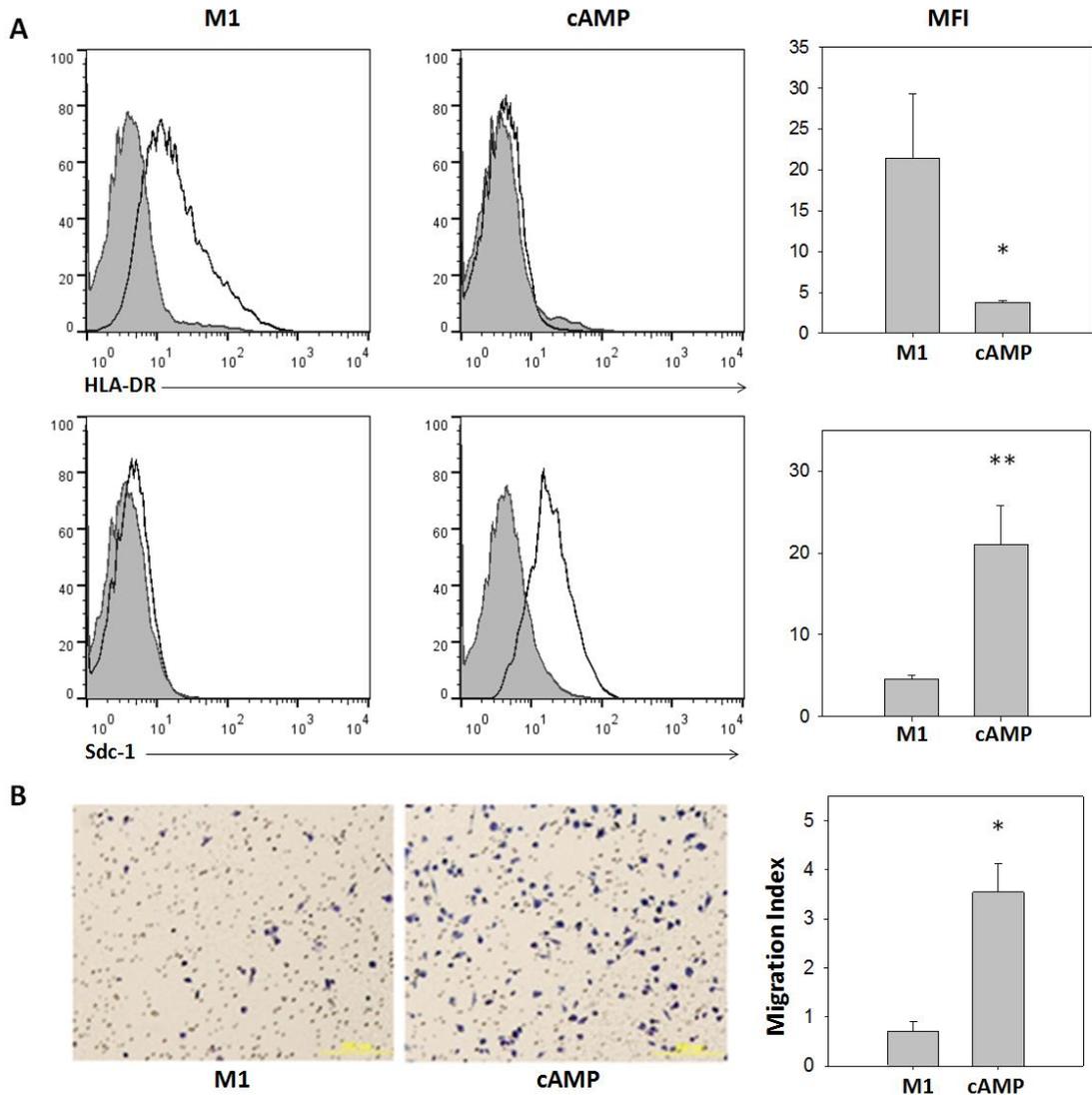


Figure 3.8. Sdc-1 expression is associated with M2 polarization state and increased motility in human macrophages. **A.** The expression of HLA-DR and Sdc-1 was analyzed on THP1 derived human macrophages following exposure to M1 stimulus (IFN- γ , 20ng/mL + LPS, 100ng/mL), or 6-benz-cAMP (100 μ M). Expression was quantified as MFI (filled: IgG control, black line: experimental; n=3-4). *P<0.05. **P<0.01. **B.** Intrinsic motility was analyzed using transwell migration to FBS. Polarized macrophages were placed into the upper chamber of a transwell and macrophages that migrated to the lower surface of the membrane was stained, counted and reported as migration index. (n=3). *P<0.05. All data represent the mean \pm SEM.

3.3 Discussion

Macrophages are being increasingly recognized as the main player in many chronic inflammatory diseases. In atherosclerosis, macrophages often represent the dominant leukocyte population in both the early and late vascular lesion [7, 8]. While mechanisms regulating atherosclerosis progression have been investigated in numerous studies, these efforts have mainly been focused on understanding monocytes recruitment into the vascular wall and the proinflammatory features of the resulting macrophages. Numerous studies have now revealed the non homogeneous nature of macrophage population during inflammation. In fact, at least four distinct macrophage polarization states, each with its own distinct biochemical signatures, have been identified *in vitro*, including M1, M2a, M2b and M2c [10, 13, 14, 129, 140]. Given the complexity of the inflammatory environment and various cytokines that are released at sites of inflammation, it is likely that more macrophage polarization states exist *in vivo*. Our investigations have identified that Sdc-1, a proteoglycan that has been reported to have anti-inflammatory effects in several inflammation models, were expressed by macrophages in both murine and human atherosclerotic lesion. Notably, this unique population of macrophages was found to display protective effects during atherosclerosis progression, which can be partly explained by their high intrinsic motility.

Within the syndecan family, Sdc-1 is emerging as an important regulator of inflammatory responses. A few studies utilizing Sdc-1^{-/-} animals have demonstrated the protective effect of Sdc-1 expression during inflammation. In a murine model of DTH and nephritis, the absence of Sdc-1 expression was associated with greater inflammatory response, prolonged inflammation, and impaired wound healing response [28, 206].

Recently, our group has also demonstrated that the absence of Sdc-1 resulted in the exacerbation of AAA formation, which was associated with increased elastin degradation and leukocyte infiltration [26]. Conversely, over expression of Sdc-1 can result in the attenuation of inflammatory responses, improved tissue healing and protection against cardiac dysfunction in murine model of myocardial infarction [27]. In agreement with these published studies, we found that Sdc-1 expression attenuated lesion formation in murine atherosclerosis model. Sdc-1 expression, which co localized with macrophages, was found to be upregulated during atherosclerosis progression, with most Sdc-1 positive cells localized to the intima and adventitia region. In contrast, little to no expression of Sdc-1 was detectable in the native aorta. Importantly, the upregulation of Sdc-1 expression and its colocalization with macrophages were also observed on human carotid plaque. Given the protective effect observed in murine atherosclerosis model, Sdc-1 expression in human macrophages may also dampen atherosclerosis progression.

While macrophages involvement during the course of atherosclerotic progression is well established and has been described in numerous publications, the effects of macrophage polarization on lesion formation has only recently been explored [221-224]. A study performed by Khallou-Laschet et al demonstrated a progressive shift in macrophage polarization during atherosclerosis progression, with M2 macrophages dominating early lesion and M1 macrophages dominating late, advanced lesions [202]. Given the contrasting functions of M1 and M2 macrophages, the ratio of M1 to M2 macrophages within atherosclerotic plaque can have a major impact in the outcome of inflammation. Our in vitro characterizations indicated that Sdc-1⁺ macrophages were not activated to M1 polarization state, but instead displayed features characteristic of M2

polarized macrophages. In fact, immunostimulated Sdc-1⁺ macrophages secreted high level of IL-10 that was comparable to M2c macrophages. Importantly, Sdc-1⁺ macrophages also displayed significantly lower leukocyte chemo-attractive potential compared to M1 polarized macrophages. Given that continuous leukocyte infiltration is one of the factors that contribute to atherosclerosis progression, Sdc-1⁺ macrophages presence can serve to limit leukocyte infiltration into the vascular lesion. Our characterizations of Sdc-1⁺ macrophages within the atherosclerotic plaque supported the notion that Sdc-1 expression is associated with M2 polarization. Immunohistochemistry analysis showed distinct pattern of iNOS and Sdc-1 expression within the vascular wall. Overall, our findings indicate that Sdc-1 expression within the atherosclerotic plaque has protective effects and may represent as one of the mechanisms through which resolution can be achieved.

In addition to macrophage polarization, macrophage motility is emerging as another factor that is crucial in determining resolution outcome [203]. Although macrophage is known to orchestrate inflammation, the notion that macrophage motility can considerably affect the outcome of inflammation has not been widely regarded previously. However, the work done by Vereyken et al has demonstrated that classically activated M1 and alternatively activated M2a macrophage displayed distinct adhesion capacity and migratory behavior toward neuronal conditioned medium, with M2a displaying a significantly higher motility response [11]. M2a macrophages were also reported to display lower adhesion to extracellular matrix compared to M1 macrophages, which was in agreement with previous report where M1 macrophages were found to display increased expression of adhesion receptors [225, 226]. In addition, van Gils et al

has also demonstrated that dampened macrophage migration can lead to the pathogenic progression of atherosclerosis, which was the result of macrophage accumulation within the vascular wall [203]. On the contrary, increased macrophage clearance and plaque regression can be achieved by stimulating macrophage migration. In agreement with these published studies, Sdc-1⁺ macrophages were found to display enhanced motility and reduced adhesion compared to M1 macrophages. In addition, Sdc-1^{-/-} macrophages were associated with dampened motility, increased adhesion and defective actin cytoskeletal reorganization. Overall, our findings suggest that the protective effect associated with macrophage Sdc-1 expression can be partly contributed to the enhanced macrophage motility during inflammation.

Thus far our findings have pointed Sdc-1 as being expressed on macrophages that display anti-inflammatory characteristics and enhanced motility. While Sdc-1 expression is restricted to noncirculating plasma cells and epithelial cells in adult tissue, expression can be induced in additional cell types, such as endothelial cells, smooth muscle cells, fibroblast and macrophages. In particular, macrophage Sdc-1 expression has been reported to be governed via cAMP/PKA dependent post-transcriptional regulation. While macrophage Sdc-1 expression can be achieved in vitro through cAMP or adenosine exposure, the biophysical process by which macrophage Sdc-1 can be regulated in vivo is unknown. Nonphlogistic clearance of apoptotic cells, or efferocytosis, is a key process in the regulation of macrophage M2 polarization state and resolution [16, 50]. Indeed, there are numerous reports detailing macrophage immune silencing consequent to efferocytosis [49, 99, 178, 179]; moreover, efferocytosis has been suggested to modulate macrophage motility and promote emigration [49]. Given our

findings, which correlate macrophage Sdc-1 expression with enhanced motility, we examined efferocytosis as an endogenous regulator of macrophage Sdc-1 expression. Indeed, we confirmed that macrophage exposure to apoptotic cells can result in the upregulation of macrophage Sdc-1 expression. This finding further supports the notion that Sdc-1⁺ macrophages are anti-inflammatory and pro resolution.

In addition to murine atherosclerosis lesion, Sdc-1 was also found to be expressed in human carotid plaque. Importantly, Sdc-1 expression appeared to colocalize with macrophages within the lesion site. In vitro characterization of Sdc-1⁺ human macrophages confirmed Sdc-1 expression was indicative of an anti-inflammatory M2 polarization state. In addition, Sdc-1⁺ human macrophages also displayed enhanced motility compared to M1 macrophages. Overall, these observations suggest that macrophage Sdc-1 is potentially be involved in the regulation of inflammation in human.

3.4 Conclusion

In summary, in this chapter we have demonstrated that Sdc-1 expression on macrophages is associated with anti-inflammatory M2 polarization and enhanced motility in both murine and human macrophages. Notably, efferocytosis was found to be a potential regulator for macrophage Sdc-1 expression in vivo. Given that the absence of a sufficient anti-inflammatory and pro resolution macrophages may serve as a driving force in the development of chronic inflammatory disease, it is important to gain a better understanding of the mechanisms regulating macrophage Sdc-1 expression during inflammation.

3.5 Methods

3.5.1 Murine Model

Both C57BL6 wild type (WT) and Apolipoprotein E (ApoE)^{-/-} mice were purchased from Jackson Laboratory. Syndecan-1 (Sdc-1)^{-/-} mice (back crossed 10 times onto a C57BL6 background) was kindly provided by Dr. Pyong Woo Park, (Baylor College of Medicine, Houston, TX). ApoE^{-/-}Sdc-1^{-/-} (C57BL6 background) double knockout mice were generated by standard crossbreeding experiments. Sdc-1^{-/-} breeder males were mated with ApoE^{-/-} females, and all mice were genotyped by PCR. All procedures were performed in male mice according to a protocol approved by Emory University and Beth Israel Deaconess Medical Center Institutional Animal Care and Use Committee.

Murine atherosclerosis model: For murine atherosclerosis model, both control and experimental (ApoE^{-/-} and ApoE^{-/-} Sdc-1^{-/-}, 8-12-week-old) group of mice were enrolled on Western high fat diet for 8 week prior to being sacrificed.

Murine peritonitis model: Sterile thioglycollate (TG) broth (6% w/v) was prepared from dehydrated TG medium (Sigma Aldrich) and sterile water. Murine peritonitis was induced by intraperitoneal (IP) injection of 0.5mL of 6% sterile TG broth.

3.5.2 Light Microscopy and Immunohistochemistry of murine atherosclerotic tissue

Following euthanization of animals, the heart and aorta were pressure perfused with 0.9% NaCl solution. Aortic tissues was harvested and embedded in n optimal cutting temperature (OCT) compound (Tissue Tek), snap frozen and 5µm transverse cryosections prepared. For histological analysis, sections were fixed in cold acetone and stained with hematoxylin and eosin. For immunohistochemical analysis, sections were

fixed in cold acetone, blocked with 10% sera from the species used to generate the secondary antibody. Immunocytochemical staining was performed using the following antibodies: goat anti Sdc-1 antibody (N-18, Santa Cruz Biotechnology) at 1:50 dilution, rat anti mouse macrophage antibody (Mac3, BD Biosciences) at 0.625µg/mL. Sections were incubated with biotinylated secondary antibodies (Vector Laboratories) followed by alkaline phosphatase streptavidin (Vector Laboratories). Negative controls with isotype IgG were prepared for each specimen. Sections were then incubated with biotinylated secondary antibodies (Vector Labs) followed by streptavidin-alkaline phosphatase (Vector Labs) at 1:500 dilution. Vector Red substrate kit 1 (Vector Labs) was used as a substrate for alkaline phosphatases. At least 4 sections from each of 3 animals at each time point in each groups were examined.

Double fluorescent immunostaining was performed to confirm colocalization of Sdc-1 with macrophages. Sections were first stained with anti Sdc-1 antibody (N-18, Santa Cruz, Biotech) at 1:50 dilution, followed by biotinylated secondary antibody and fluorescein conjugated streptavidin (Vector Labs) at 7.5 µg/mL. To detect macrophages, the sections were then incubated with anti Mac3 antibody (BD Biosciences) at 1:50 dilution, followed by Alexa 594 conjugated secondary antibody (Molecular Probes). Nuclei were visualized by 4',6-diamidino-2-phenylindole (DAPI) staining. At least 4 sections from each of 3 mice at each time point from each genotype were examined.

Plaque iNOS was analyzed by staining with rabbit anti-mouse iNOS antibody (Abcam), detected by biotinylated goat anti-rabbit secondary and standard streptavidin-HRP/Diaminobenzidine (DAB). Quantification of iNOS positive plaque area was

calculated using Image Pro Plus. At least 4 sections from each of 3 mice from each genotype were examined.

3.5.3 Light Microscopy and Immunohistochemistry of human atherosclerotic tissue

Specimens consisting of human atherosclerotic tissue were collected at Emory University Hospital under an approved IRB protocol. Specimens were fixed in 10% formalin overnight and then processed in a paraffin block. 5 μ m thick serial sections were analyzed with hematoxylin and eosin (Richard-Allan Scientific). Immunocytochemical staining was performed to identify Sdc-1 and macrophages. Fifteen tissue sections from each of five tissue samples were examined. The following antibodies were used: monoclonal mouse anti human CD68 (PG-M1, Dako) at 1:100 dilution for macrophage identification, monoclonal Sdc-1 (DL101, Santa Cruz) at 1:100 dilution for Sdc-1 identification. Mouse IgG1 κ (M9035, Sigma Aldrich) was used for negative control. The avidin-biotin complex (ABC) method was used for staining. After deparaffinized and rehydrated, sections were incubated in water bath for 30 minutes at 95°C with target retrieval solution (S1700, Dako) for antigen retrieval. Sections were blocked with avidin and biotin blocking solutions (X0590, Dako) and 10% goat serum. The sections were incubated with primary antibodies for 60 minutes at room temperature, washed and incubated for 30 minutes with a biotinylated goat anti mouse IgG secondary antibody at 1:1500 dilution. After washing, the slides were incubated with streptavidin-HRP (KO679, Dako) for 30 minutes, then DAB substrate solution (KO679, Dako) was added yielded a brown product on positive cells. Slides were counterstained with a hematoxylin and mounted with xylene based mounting medium (Richard-Allan Scientific).

3.5.4 Cell culture and activation

Murine peritoneal macrophages: Mature peritoneal macrophages were isolated from the peritoneal exudates at 4 or 5 days following peritonitis initiation. Peritoneal macrophages were cultured in Dulbecco's Modified Eagle Medium (DMEM) (Cellgro) containing 10% FBS (Cellgro), 100U/mL penicillin and 100µg/ml streptomycin sulfate (Sigma Aldrich) at 37°C in 5% CO₂ humidified incubator. Where indicated, macrophage activation was induced by culturing macrophages overnight with Ifn-γ (100U/mL) and LPS (100ng/mL) for M1 polarization, IL-4 (20ng/mL) for M2a polarization, and IL-10 (100ng/mL) for M2c polarization (all from R&D Systems). Sdc-1 expression was induced by culturing macrophage overnight with 6-Bnz-cAMP (Sigma Aldrich, 100µM) or Adenosine (Sigma Aldrich, 375 µM). Following activation period, macrophages were harvested using non-enzymatic cell dissociation buffer (Sigma Aldrich), washed twice with PBS, counted and used in further analysis/experiments as indicated.

Murine RAW 264.7 macrophages: Murine RAW264.7 macrophages were cultured in DMEM (Cellgro) containing 10% FBS (Cellgro), 100U/mL penicillin and 100µg/ml streptomycin sulfate (Sigma Aldrich) at 37°C in 5% CO₂ humidified incubator. Cell culture was performed in 100x200mm tissue culture dish. Macrophages were obtained by scraping and were passaged twice weekly. For experiments, macrophages were obtained by scraping before being washed twice in PBS, counted and used in further analysis/experiments as indicated.

Human THP1 monocytes: Human acute monocytic leukemia cell line was obtained from ATCC and cultured in suspension in Roswell Park Memorial Institute (RPMI) media (ATCC), supplemented with 10% FBS (ATCC) and 50nM 2-mercaptoethanol (Sigma Aldrich). Cell culture was performed at 2x10⁵ cells/mL in tissue

culture flasks and cells were passaged twice weekly when the concentration reached 1×10^6 cells/mL. Monocytic differentiation was induced by incubation of 1×10^7 cells in 100x200mm tissue culture dish in culture media that has been supplemented with 50ng/mL of phorbol 12-myristate 13-acetate (PMA) (Sigma Aldrich) for 3 days. After the differentiation period, M1 polarization was induced by culturing macrophages overnight with Ifn- γ (20ng/mL) and LPS (100ng/mL), while Sdc-1 expression was induced by culturing macrophage with 6-Bnz-cAMP (100 μ M) for 40 h.

3.5.5 Flow cytometry

Isolated macrophages for surface marker expression analysis were passed through a 70-micron cell strainer to remove debris and then counted and resuspended in FACS buffer (1% bovine serum albumin (BSA) in PBS) at a concentration of 5×10^6 /mL. Staining was performed by combining the manufacturer's recommended quantity of primary antibody to 100 μ L of cell suspension, followed by 30 minutes of incubation at 4°C. Cells were washed twice before being analyzed on the flow cytometer (LSRII, Becton Dickinson).

The cell antibodies used for mouse cell staining included: anti-CD3 (145-2C11), anti-F4/80 (BM8), anti-I-A/I-E (M5/114.15.2), anti-CD86 (GL1), anti-CD273/PD-L2 (TY25), anti-CD138/Sdc-1 (281-2) and anti-CD184/CXCR4 (2B11); negative controls with isotype IgG were included for each marker. Anti-F4/80 and anti CD273 antibodies were obtained from eBioscience and the rest were obtained from BD Biosciences.

The cell antibodies used for human cell surface markers analysis included: anti-HLA-DR (G46-6), anti-CD163 (GHI/61), anti-CD138/Sdc-1 (MI15) and anti-

CD184/CXCR4 (12G5); negative controls with isotype IgG were included for each marker. All of the antibodies were obtained from BD Biosciences.

3.5.6 Nitrite Assay

Generation of nitrites was measured using Griess reagent kit (Invitrogen) according to manufacturer's protocol. Briefly, conditioned media were collected from polarized macrophages that have been cultured for 18 hours. The nitrite level was measured from 150 μ L of filtered conditioned media (0.22 μ m filter) from each sample and it was compared to standard curve generated using standard nitrite solutions.

3.5.7 Arginase Assay

Arginase activity level was determined as previously published. Briefly, 4×10^5 macrophages were lysed with 80 μ L 0.1% Triton X-100 (Sigma Aldrich). 20 μ L of Tris-HCL and MnCl₂ mixture was then added to the lysates to a final concentration of 25mM and 1mM respectively. Arginase was activated by heating at 55°C for 10 minutes and 100 μ L of L-arginine solution was then added to the mixture to a final concentration of 50mM. Mixtures were incubated at 37°C for 60 minutes and stopped with the addition of 800 μ L H₂SO₄(96%)/H₃PO₄(85%)/H₂O (1/3/7, v/v/v). 40 μ L of α -isonitrosopropiophenone (9% solution in EtOH) was then added, followed by heating at 100°C for 30 minutes. Urea production was measured by measuring absorbance at 540 nm and compared to a standard curve. Arginase activity was compared to control macrophages (unstimulated peritoneal macrophages) and reported as fold change.

3.5.8 Chemokine Profile

For IL-10 ELISA, macrophages were first plated at 10×10^6 cells per 100x20mm tissue culture dish in serum free DMEM. Macrophage M1, M2a and M2c polarization

and macrophage Sdc-1 expression (Ado) was induced as described above with the addition of LPS at 100ng/mL. With the exception of M2c polarization, conditioned media was collected after 24 hours. For M2c polarization, macrophages were cultured in the presence of the stimulus and LPS for 24 hours. After the initial stimulation, serum free DMEM was added and conditioned media was collected after an additional 24 hours of culture. Collected media was filtered (0.22µm) and subjected to 8-fold concentration (Amicon Ultra, 3000 NMWL) and analyzed using an IL-10 ELISA kit (SA Biosciences).

3.5.9 Phagocytosis Index

Peritoneal macrophages were cultured in a 6 well cell culture plate (2×10^6 /well) and macrophage polarization was induced as described above. % phagocytosis was determined by incubating macrophages with fluorescently labeled microspheres (1µm diameter, Polysciences) at a ratio of 1:15 for 2 hours at 37°C in 5% CO₂ humidified incubator. Excess microspheres were removed by washing 3x with PBS and macrophages were then harvested using non-enzymatic cell dissociation buffer (Sigma Aldrich) and washed. Percent phagocytosis was obtained by analyzing the macrophages using flow cytometry.

3.5.10 PCR Array

mRNA expression analysis of 84 genes that encode chemokines and their receptors were performed using mouse chemokines and receptors RT2 profiler PCR array (SABiosciences). RNA was extracted from classically activated M1 and Sdc-1⁺ (Adenosine) macrophages with TRIzol reagent (Invitrogen) and purified using RNeasy mini kit (Qiagen) according to manufacturer's protocol. A total of 1µg of RNA from each sample were converted to cDNA using the RT² first strand kits (SABiosciences) and

was then subjected to PCR array analysis on the Applied Biosystems 7900 using the RT² qPCR Master Mixes according to manufacturer's protocol (SABiosciences). Data were analyzed using the web-base PCR array data analysis tool from SABiosciences. mRNA expression level for each gene was normalized to the expression level of glyceraldehydes-3-phosphage dehydrogenase (GAPDH) using the equation $2^{-(Ct[\text{gene of interest}] - Ct[\text{GAPDH}])}$, where Ct is the threshold cycle. Normalized gene expression levels of experimental samples (Sdc-1⁺ macrophages) were obtained by comparing the gene expression level to control samples (M1 macrophages). Each reported value represents the mean fold increase or fold decrease of mRNA expression in experimental samples relative to controls.

3.5.11 Leukocyte Chemotaxis

Peripheral mouse blood monocytes were obtained by negative selection using the Mouse Monocytes Enrichment Kit (StemCell Technologies) according to manufacturer's instruction. Briefly, mouse blood was obtained through cardiac puncture and was collected in syringes and tubes coated with sodium citrate. Leukocytes were obtained following red blood cell (RBC) lysis using RBC Lysis Buffer (eBioscience) and PBS wash. Mouse monocytes were obtained from the cell suspension using the Mouse Monocytes Enrichment Kit (StemCell Technologies) according to manufacturer's instruction.

CD4⁺ T cells were negatively selected from splenocytes (Miltenyi Biotech) according to manufacturer's protocol. Enriched T cells were cultured in DMEM supplemented with 10% FBS, IL-2 (10ng/mL, R&D Systems), IL-12 (20ng/mL, R&D Systems) to produce the desired Th1 polarized response, while Dynabeads Mouse T-

Activator CD3/CD28 (Invitrogen) was utilized for the activation and expansion of the T cells according to manufacturer's protocol.

Chemotaxis assays were performed by loading 100 μ L of T cells or monocytes that have been resuspended at a concentration of 1×10^5 cells/mL in DMEM containing 0.5% FBS, onto the upper well of a 24-well transwell permeable supports with a pore size of 5 μ m (Corning). Conditioned media obtained (600 μ L) from polarized macrophages that have been cultured for 18 h in DMEM containing 0.5% FBS was added to the lower well. The migration response was quantified after 4 hours of incubation at 37°C in 5% CO₂ humidified incubator and was reported as % migrated.

3.5.12 Macrophage Migration Assay

Intrinsic motility of macrophages was analyzed by loading 100 μ L of peritoneal macrophages that have been resuspended at a concentration of 1×10^6 /mL in DMEM containing 0.5% FBS onto the upper well of a 24-well transwell permeable supports with a pore size of 8 μ m (Corning). In experimental group, DMEM containing 10% FBS was added onto the lower well, and macrophages were then incubated at 37°C in 5% CO₂ humidified incubator; DMEM containing 0.5% FBS was used in the control group. In order to analyze the intrinsic motility of polarized macrophages, M1, M2a, M2c or cAMP was added onto both upper and lower chambers. After 18 hours of incubation, macrophages that have migrated onto the lower well surface of the membrane were fixed with 10% formalin in PBS for 10 minutes. Macrophages that adhere to the upper well were scraped and the membrane was then stained with hematoxylin (Sigma Aldrich). For each replicate, peritoneal macrophages were obtained from separate mice and 3 separate wells were included. The numbers of macrophages in three independent fields of view

were counted in each well and averaged. Migration was quantified as migration index by dividing the number of macrophages counted in experimental compared to control group.

Intrinsic motility of human THP1 derived macrophages was investigated in a similar manner as described above. However, only the effect of M1 stimulus and cAMP on macrophage migration response was analyzed.

3.5.13 Adhesion Assay

Macrophage adhesion to fibronectin was determined using macrophages that have been activated with M1 stimulus or 6-Bnz-cAMP and were labeled with 1.5 μ M CellTrace carboxyfluorescein succinimidyl ester (CFSE) (Invitrogen) according to manufacturer's protocol. Following fluorescent labeling, cells were harvested, washed and seeded (1×10^5 in 100 μ L) in 96 wells cell plate that has been coated with fibronectin (5 μ g/cm²). Macrophages were then cultured for 2 hours at 37°C in 5% CO₂ humidified incubator. Non-adherent macrophages were removed by washing 3 times with PBS and adherent macrophages were then lysed with 100 μ L of 0.1M NaOH. Fluorescence signal was measured using a fluorescence plate reader. Cell number was obtained using a standard curve generated with known concentration of fluorescently labeled macrophages.

3.5.14 Actin Staining

Cultured Sdc-1^{+/+} or Sdc-1^{-/-} macrophages were fixed with fixed with 10% formalin in PBS for 10 minutes and permeabilized with PBS containing 0.5% Triton X-100 (Sigma Aldrich), rinsed with 100mM glycine (Sigma Aldrich), and incubated with block buffer (0.2% TrionX-100 and 6% goat serum in PBS). Cells were then stained with Alexa Fluor568 conjugated Phalloidin (Invitrogen). Nuclei were counterstained with DAPI (Invitrogen) prior to being evaluated with confocal microscopy.

3.5.15 Generation of Apoptotic Cells and Efferocytosis Experiment

Apoptotic RAW 264.7 cells macrophages were generated by exposure of plated macrophages to ultraviolet (UV) light. Macrophages were plated at 10×10^6 cells per 100x20mm tissue culture dish and were then placed 5 inches from the UV (254nm) source and exposed to UV light for 5 minutes with the culture dish lid off in a cell culture hood. Irradiated macrophages were then cultured at 37°C in 5% CO₂ humidified incubator for 2 hours. Apoptotic macrophages were then harvested using non-enzymatic cell dissociation buffer (Sigma Aldrich), washed using PBS prior to being used.

Efferocytosis experiment was performed by adding apoptotic macrophages to cultured peritoneal macrophages (15 to 1 ratio) in DMEM supplemented with 0.5% FBS, which were then placed at 37°C in 5% CO₂ humidified incubator. Following 2 hours of incubation, excess apoptotic cells were removed by washing with PBS and remaining macrophages were cultured for additional 40 hours. Macrophages were removed using harvested using non-enzymatic cell dissociation buffer (Sigma Aldrich) and washed using PBS prior to being stained for flow cytometry analysis.

3.5.16 Statistical Analysis

Means and SEM were calculated for each parameter. All data were analyzed via 2-tailed Student *t* test. For the PCR array analysis, the P values were calculated based on a Student *t* test of the replicate $2^{-(Ct[\text{gene of interest}] - Ct[\text{GAPDH}])}$ values for each gene in both the control and experimental groups. Values of $P < 0.05$ were considered statistically significant.

CHAPTER 4

SYNDECAN-1 PARTICIPATES IN MACROPHAGE EGRESS TOWARD THE LYMPHATIC NETWORK

Our findings have established that Sdc-1 expression on macrophages is indicative of an anti-inflammatory polarization state and that its presence during inflammation can dampen pathogenic disease progression. Our characterizations suggest that in addition to the anti-inflammatory macrophage polarization state, the observed protective effect can also be potentially explained by enhanced macrophage migration associated with Sdc-1 expression. In fact, one of the key features that define resolution of inflammation is the effective egress of macrophages out of the inflammatory site to the draining lymphatic network. Given our findings, we postulate that the increased motility response observed in Sdc-1⁺ macrophages can contribute to increased macrophage egress from inflammation site, which results in dampened inflammatory responses and resolution of inflammation.

4.1 Introduction

One of the defining features of chronic inflammation is the persistence of activated macrophages at the site of inflammation. While numerous studies have confirmed that continuous leukocyte influx can contribute to the development of chronic inflammation, failure of macrophages to egress from the site of inflammation to the draining lymphatics is another factor that has been shown to participate in the process [7, 16, 41]. This delayed macrophage clearance can result in the accumulation of activated macrophages, which results in the maintenance of proinflammatory environment within

the inflammation site. This, in turn, can result in the additional recruitment of monocytes that mature into proinflammatory macrophages within the tissue. Overall, delayed macrophage clearance can result in the generation of a proinflammatory feedback loop that fuels the development of chronic inflammation.

Studies utilizing various murine inflammation models, including atherosclerosis, lung inflammation and peritonitis have demonstrated that macrophages can exit inflammation site via nearby draining lymphatics during inflammation [51, 99, 128, 198, 227]. Importantly, egress toward the lymphatics was associated with inflammation resolution [49, 99, 198]. In murine model of atherosclerosis regression, surgical transplantation followed by aggressive lowering of cholesterol level resulted in macrophage clearance to the nearby draining lymph nodes, which was associated with significant decrease in atherosclerotic plaque size [198]. A similar process involving macrophage migration toward the lymphatics has also been noted in the resolution of peritonitis, where macrophages were observed to egress from the peritoneal cavity toward the draining lymph nodes [51, 99]. In addition, macrophage efferocytosis was found to accelerate macrophage clearance toward the draining lymphatics [99]. Overall, these reports indicate that macrophage egress into the draining lymph nodes is correlated with inflammatory resolution.

In the previous chapter, we have established that Sdc-1 expression on macrophages is associated not only with anti-inflammatory polarization state, but also with enhanced motility response. In addition, absence of Sdc-1 expression within the atherosclerotic plaque was associated with accelerated plaque growth. Thus, we hypothesized that the enhanced macrophage motility associated with Sdc-1 expression

can contribute to macrophage egress from the site of inflammation and thus limit the progression of inflammation. In fact, the link between macrophage Sdc-1 expression and migration toward the lymphatic has been suggested previously [29], though it was not confirmed.

In this chapter, we establish that the enhanced motility associated with Sdc-1 expression is indeed associated with macrophage egress from the inflammation site toward the draining lymphatics. Characterization of macrophage influx and efflux from the peritoneal cavity during peritonitis in both Sdc-1^{+/+} and Sc-1^{-/-} mice revealed that the absence of Sdc-1 expression resulted in the accumulation of macrophages within the peritoneal cavity and delayed macrophage clearance. In addition, adoptive transfer of fluorescently labeled Sdc-1^{+/+} and Sc-1^{-/-} macrophages into the peritoneal cavity of mice undergoing peritonitis confirmed that Sdc-1 absence resulted in decreased macrophage egress toward the draining lymphatic. Thus, our data suggest that the protective effects associated with Sdc-1 expression can be attributed to enhanced macrophage egress, which supports the resolution of inflammation

4.2 Results

4.2.1 Sdc-1 is expressed on macrophage during the course of peritonitis response

The potential involvement of Sdc-1 in macrophage clearance during inflammation was investigated using a murine model of peritonitis, a self resolving model of acute inflammation that is commonly used to study leukocyte clearance to the draining lymph nodes. In order to determine Sdc-1 involvement during peritonitis, flow cytometry was used to characterize Sdc-1 expression in concert with macrophage infiltrate over the 12 day time course of TG induced murine peritonitis. Flow cytometry analysis confirmed

that Sdc-1 was expressed on macrophages during the 12 day time course of peritonitis. The number of Sdc-1⁺ macrophages increased and peaked 2 days after the peritonitis induction, and decreased thereafter (Fig.4.1A), while little to no expression of Sdc-1 was detected on resident peritoneal macrophages. It is worth noting that not all of the macrophages express Sdc-1, which highlighted the non homogeneous nature of the macrophages present in the peritoneal cavity during peritonitis (Fig.4.1B). In light of our in vitro characterization of Sdc-1⁺ macrophages, which showed the relationship between Sdc-1 expression and enhanced motility, we sought to compare the kinetics of Sdc-1⁻ and Sdc-1⁺ macrophages during the peritonitis response. We observed that early during the peritonitis response (12hr), only a small portion of the macrophages are expressing Sdc-1 (Fig.4.2A). As the peritonitis progressed, the number of Sdc-1⁻ and Sdc-1⁺ macrophages was not significantly different, though both increased and peaked at day 2. Significantly, the decline in the total macrophage population thereafter was associated with a dominant loss of Sdc-1⁺ compared to Sdc-1⁻ macrophages (Fig.4.2A). Overall, our findings confirm the involvement of macrophage Sdc-1 in the regulation of murine TG induce peritonitis model.

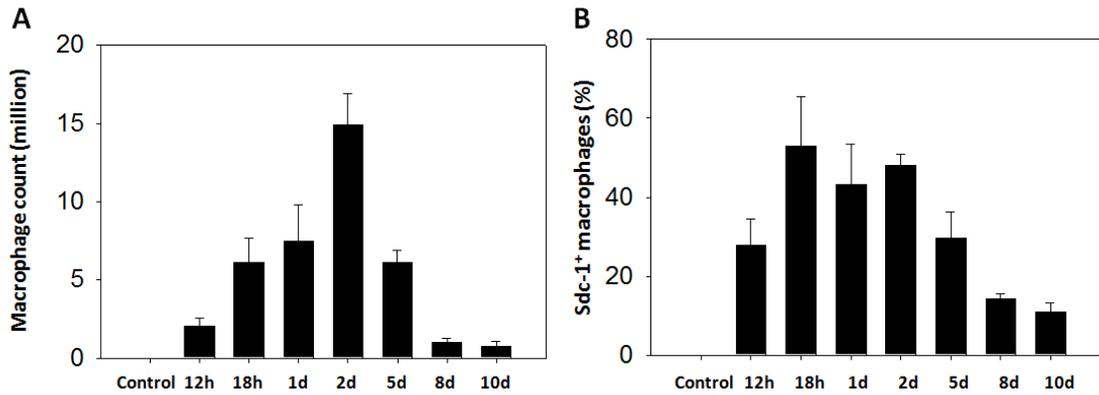


Figure 4.1. Macrophage Sdc-1 expression during the course of peritonitis. **A.** Macrophages response was analyzed at various time points post thioglycollate injection. **B.** Macrophage response in conjunction with Sdc-1 expression was analyzed at various time points. % of Sdc-1 expressing macrophages compared to total macrophages was analyzed at various time points during peritonitis response (n=3, 5, 5, 3, 3, 4, 3, 8 at 0, 12 hours (h), 18h, 1day (d), 2d, 5d, 8d and 10d respectively). All data represent the mean \pm SEM.

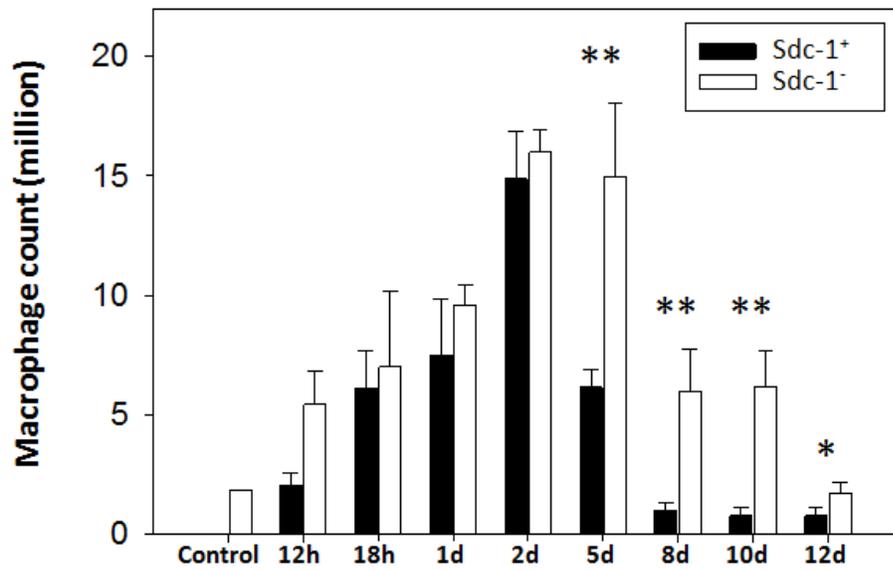


Figure 4.2. Macrophage Sdc-1 expression during the course of peritonitis. Macrophage response in conjunction with Sdc-1 expression was analyzed at various time points post thioglycollate injection (n=3, 5, 5, 3, 3, 4, 3, 8, and 4 at 0, 12 hours (h), 18h, 1day (d), 2d, 5d, 8d, 10d and 12d respectively). *P<0.05. **P<0.01. All data represent the mean \pm SEM.

4.2.2 The absence of Sdc-1 expression is associated with macrophage accumulation within the peritoneal cavity

To further study the functional significance of Sdc-1 in macrophage egress during inflammation, we enrolled both Sdc-1^{+/+} and Sdc-1^{-/-} mice in the TG induced peritonitis model and compared the leukocyte influx and efflux profile. Cells were harvested by lavage over the 12 day time course model, total cell counts were recorded and flow cytometry was utilized to identify neutrophils and macrophage populations. While analysis of total cell counts revealed no difference in the kinetics of inflammatory influx and the magnitude of the peritonitis response, as noted in the peak of the response at day 2, we did observe a significant difference at later time points, with greater cell numbers observed in Sdc-1^{-/-} mice (Fig.4.3A). In addition, we also analyzed the percent neutrophils and percent macrophages at each time point. The evolution and resolution of the neutrophil response was characteristic of a self resolving peritonitis model with no differences observed between Sdc-1^{+/+} and Sdc-1^{-/-} mice (Fig.4.3B). Looking at the macrophages, influx response was found to be similar in Sdc-1^{+/+} and Sdc-1^{-/-} mice. However, we did record a significant difference in the resolution of the macrophage response, with greater number of macrophages remaining in the peritoneal cavity of Sdc-1^{-/-} mice (Fig.4.3C,D). Thus, similar to our previous in vitro characterization, our findings from the peritonitis model suggest that Sdc-1 expression may be an important modulator in macrophage emigration.

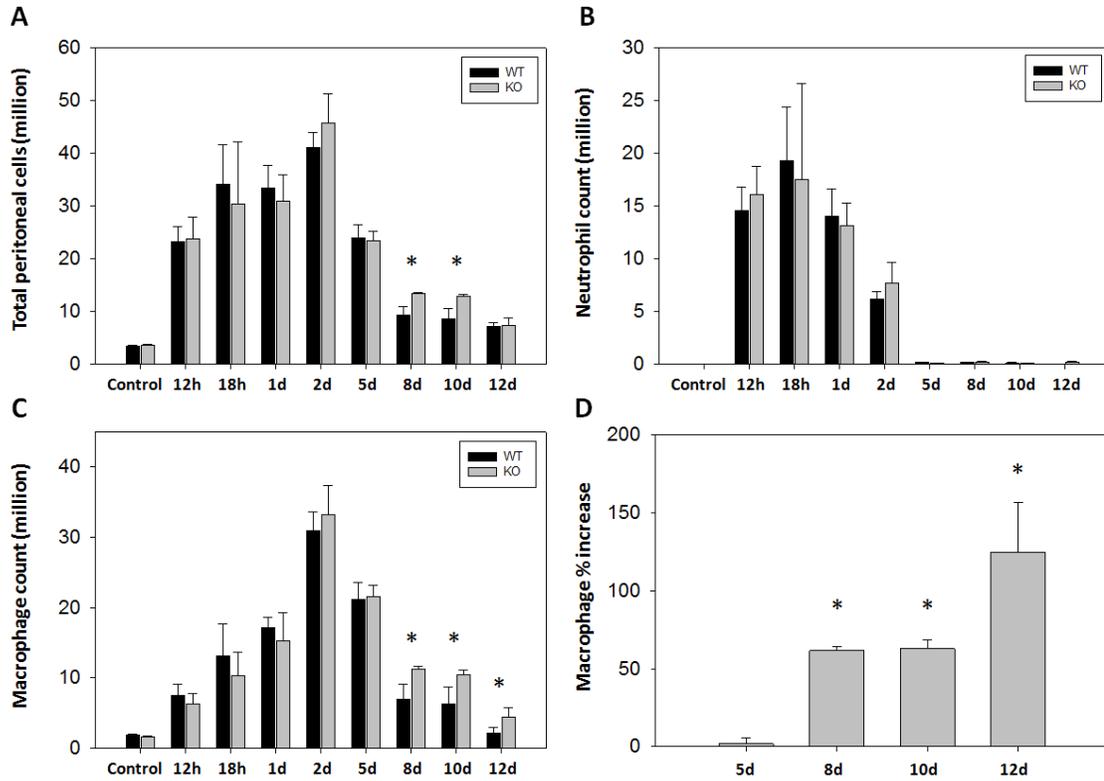


Figure 4.3. Leukocyte response during the course of peritonitis. **A-C.** Leukocytes response was analyzed in thioglycollate induced peritonitis in *Sdc-1*^{+/+} (WT) and *Sdc-1*^{-/-} (KO) mice. **A.** Total peritoneal cells, **B.** neutrophils, and **C.** macrophages response was analyzed at various time points post thioglycollate injection. **D.** % increase of KO compared to WT macrophages that remain in peritoneal cavity at 5,8,10 and 12 days post thioglycollate injection (n=3, 5, 5, 3, 3, 4, 3, 8 and 4 at 0, 12 hours (h), 18h, 1day (d), 2d, 5d, 8d and 10d respectively). All data represent the mean \pm SEM.

4.2.3 Sdc-1 expression is involved in macrophage egress toward the draining lymph nodes during inflammation resolution

One of the hallmarks of chronic inflammatory diseases is macrophage failure to egress from the inflammation site to the draining lymph nodes. As described above, the absence of Sdc-1 expression was found to result in macrophage accumulation within the peritoneal cavity. In addition, we have also demonstrated that Sdc-1 absence was associated with increased atherosclerotic lesion formation (Chapter 3). Given the association between the lack of Sdc-1 expression and reduced macrophage intrinsic motility, it is probable that the delayed macrophage clearance observed in Sdc-1^{-/-} mice is related to dampened macrophage egress toward the draining lymph nodes. To investigate this question, we utilized an adoptive transfer model in which fluorescently labeled macrophages were transferred into the peritoneal cavity of mice undergoing the same stage of peritonitis. In order to verify Sdc-1 involvement in macrophage clearance to the draining lymph nodes, Sdc-1^{+/+} and Sdc-1^{-/-} macrophages were fluorescently labeled with CellTrace carboxyfluorescein diacetate succinimidyl (CFSE) and CellTrace Violet respectively, and mixed at a ratio of 1:1. The fluorescently labeled mixed macrophage population was then adoptively transferred into the peritoneal cavity of Sdc-1^{+/+} mice undergoing peritonitis at the same stage. Mesenteric lymph nodes and spleen were collected following 18 hours of incubation period. Leukocyte suspension obtained from processed tissue was analyzed using flow cytometry analysis to check for the presence of adoptively transferred labeled macrophages. Consistent with our in vitro finding that Sdc-1 absence resulted in decreased intrinsic motility, deficiency in macrophage Sdc-1 expression resulted in dampened macrophage egress toward the draining lymphatic.

Flow cytometry analysis of fluorescently labeled macrophages recovered from processed tissue showed higher percentage of Sdc-1^{+/+} macrophages compared to Sdc-1^{-/-} macrophages in both the mesenteric lymph nodes and spleen (Fig.4.4). These findings suggest that dampened macrophage intrinsic motility observed in vitro in the absence of Sdc-1 expression can translate to reduced macrophage motility and egress in vivo. In addition, these results reinforce the significance of macrophage motility as a regulator of inflammatory response.

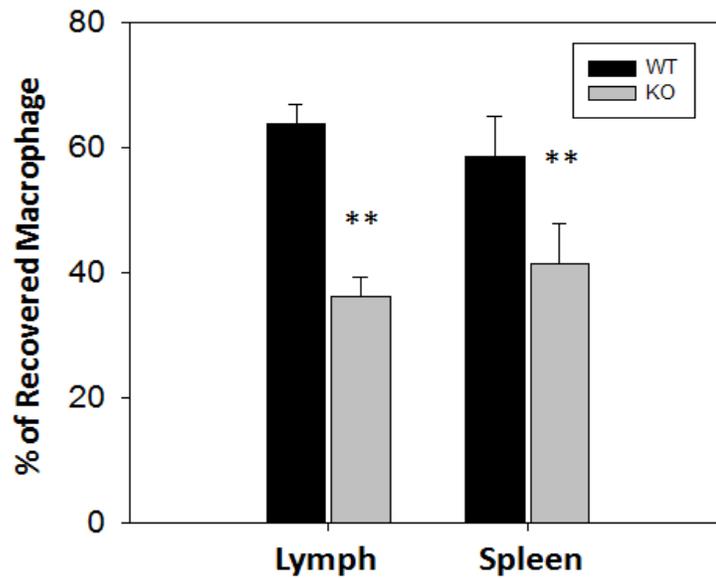


Figure 4.4. Absence of Sdc-1 expression is associated with delayed macrophage clearance to the lymphatics. Fluorescently labeled Sdc-1^{+/+} (WT) and Sdc-1^{-/-} (KO) macrophages (1:1 mixture) were adoptively transferred into the peritoneal cavity of WT mice undergoing the same stage of peritonitis (day 3 post peritonitis induction). Mice were sacrificed at 18 hours post adoptive transfer and the mesenteric lymph nodes and spleen were harvested and processed. Cells obtained from the tissues were analyzed to determine the percentage of WT and KO macrophages from fluorescently labeled cells that were recovered (n=4). **P<0.01. All data represent the mean ± SEM.

4.3 Discussion

Macrophage egress is understood to be crucial for the effective resolution of inflammation and numerous recent publications have attempted to uncover the stimulus, receptors, signaling pathways and other points of control that are involved in this process [41, 49, 51, 90, 99, 128, 198, 199, 203]. While progress has been made in understanding the regulation of macrophage clearance, many of the mechanisms involved are still poorly understood. In this chapter, we report that the absence of macrophage Sdc-1 expression does not affect macrophage influx within the peritoneal cavity during peritonitis. Instead, Sdc-1 was found to be involved in the egress of macrophages from the peritoneal cavity into the local draining lymphatic. Thus, macrophage Sdc-1 expression during inflammatory response may serve to limit the scope of inflammation through active macrophage clearance out of the inflamed tissue.

As we have demonstrated in the previous chapter, the absence of Sdc-1 expression on macrophages was associated with dampened motility and exaggerated atherosclerosis progression. While macrophage egress to the lymphatic has been previously studied using murine model of atherosclerosis, it involved robust lipoprotein profile change achieved through surgical transplantation of atherosclerotic arterial segment or ApoE gene complementation using adenoviral vectors [41, 198]. In an attempt to investigate the mechanism by which increased macrophage motility can lead to protective effect during inflammation, we investigated macrophage infiltration and emigration in a murine peritonitis model. In agreement with our previous finding in which macrophage Sdc-1 expression was found to be upregulated in response to efferocytosis, we observed an upregulation of macrophage Sdc-1 expression that

coincided with neutrophils infiltration and clearance. Unlike macrophages, neutrophils quickly turn apoptotic following extravasation to the inflammation site and are subsequently cleared by infiltrating macrophages through efferocytosis. We observed that the neutrophil count was significantly upregulated at 12 hours and reached a maximum at 18 hours post TG injection. Accordingly, the number of Sdc-1⁺ macrophage within the peritoneal cavity increased significantly during this time frame. Thereafter, the number of neutrophils started to decrease, with the majority of neutrophils being cleared 2 days post TG injection. At the same time, the number of Sdc-1⁺ macrophages continued to increase within this time frame. Interestingly, the number of Sdc-1⁺ macrophages started to decrease following neutrophils absence in the peritoneal cavity. Thus, it is likely that macrophage Sdc-1 expression during peritonitis was associated with macrophage efferocytosis of apoptotic neutrophils. In addition, it is important to note that the early decline in total macrophage count from the peritoneal cavity was largely associated with the loss of macrophages that express Sdc-1. Decrease in the number Sdc-1⁻ macrophages was noted only following the clearance of the majority of Sdc-1⁺ macrophages from the peritoneal cavity. Overall, our data support our previous finding, which linked macrophage Sdc-1 expression with efferocytosis and enhanced motility.

Our in vivo data support the notion that Sdc-1 expression is involved in macrophage clearance during inflammation. Indeed, further characterization utilizing Sdc-1^{-/-} mice confirmed that while the total macrophage influx was similar to Sdc-1^{+/+} mice, the absence of Sdc-1 expression was associated with delayed macrophage clearance out of the peritoneal site. While the reduction that we observed was modest, the difference recorded was statistically significant. In addition, given the acute nature of

peritonitis model, it is possible that under a stronger inflammatory challenge, a more profound difference in macrophage clearance can be obtained. Nevertheless, in vivo adoptive transfer of fluorescently labeled Sdc-1^{+/+} and Sdc-1^{-/-} mixed macrophage population confirmed that Sdc-1 absence resulted in significantly decreased egress toward the draining lymph node and spleen. Thus, delayed macrophage clearance from the peritoneal cavity in the absence of Sdc-1 expression can be correlated with dampened macrophage egress toward the lymphatic system. This suggests that macrophage Sdc-1 expression is potentially a part of the mechanisms that is involved in the regulation of inflammation resolution.

4.4 Conclusion

In summary, in this chapter we have demonstrated that Sdc-1 expression was involved in macrophage clearance from the site of inflammation. Notably, Sdc-1 expression was also associated with enhanced macrophage egress toward the draining lymph nodes. Overall, these findings reinforce the physiological significance of macrophage Sdc-1 expression during inflammatory response and their involvement in the resolution of inflammation.

4.5 Method

4.5.1 Murine Peritonitis Model

C57BL6 wild type (WT) mice were purchased from Jackson Laboratory. Syndecan-1 (Sdc-1)^{-/-} mice (back crossed 10 times onto a C57BL6 background) was kindly provided by Dr. Pyong Woo Park, (Baylor College of Medicine, Houston, TX). All procedures were performed in male mice according to a protocol approved by Emory University and Beth Israel Deaconess Medical Center Institutional Animal Care and Use Committee.

Sterile TG broth (6% w/v) was prepared from dehydrated TG medium (Sigma Aldrich) and sterile water. Murine peritonitis was induced by IP injection of 0.5mL of 6% sterile TG broth. For characterization of peritonitis response, peritoneal exudates were collected at 12 hours, 18 hours, 1 day, 2 days, 5 days, 8 days, 10 days, and 12 days following peritonitis induction. Peritoneal leukocytes were obtained through lavage using 5mL of sterile PBS with 1mM EDTA. The expression of Sdc-1 and the number of macrophages, neutrophils, and T cells in the peritoneal lavage was determined by performing total cell count and analyzing the percentages of each cell types using flow cytometry analysis.

4.5.2 Flow cytometry

Isolated macrophages for surface marker expression analysis were passed through a 70-micron cell strainer to remove debris and then counted and resuspended in FACS buffer (1% BSA in PBS) at a concentration of 5×10^6 /mL. Staining was performed by combining the manufacturer's recommended quantity of primary antibody to 100 μ L of

cell suspension, followed by 30 minutes of incubation at 4°C. Cells were washed twice before being analyzed on the flow cytometer (LSRII, Becton Dickinson).

The cell antibodies used mouse cell staining included: anti-Gr1 (RB6-8C5), anti-CD3 (145-2C11), anti-F4/80 (BM8), and anti-CD138/Sdc-1 (281-2); negative controls with isotype IgG were included for each marker. Anti-F4/80 antibodies were obtained from eBioscience and the rest were obtained from BD Biosciences.

4.5.3 Adoptive Transfer

For adoptive cell transfer experiments, peritonitis was induced in both WT and Sdc-1^{-/-} donor mice and WT recipient mice 3 days prior to adoptive transfer. WT macrophages were labeled with 3µM CellTrace CFSE (Invitrogen) while Sdc-1^{-/-} macrophages were labeled with 5 µM CellTrace Violet (Invitrogen) according to manufacturer's protocol. WT and Sdc-1^{-/-} macrophages were then counted and mixed (1:1 ratio) at a final concentration of 1x10⁶ cells/mL in sterile PBS. Mixed cell suspension was then injected into the peritoneal cavity of WT recipient mice (1mL/mice) that were undergoing the same stage of peritonitis as the donor mice. Recipient mice were sacrificed 18 hours post adoptive transfer and the mesenteric lymph nodes and spleen were harvested and processed for flow cytometry analysis. Lymph node was processed by mechanical disruption. Lymph nodes were first gently disaggregated with the tip of 18G needles in a small petri dish with DMEM supplemented with 5% FBS, before being further processed by pressing against the bottom of the petri dish with the plunger of a 6 mL syringe in a circular motion until mostly fibrous tissue remains. Tissue clumps were further dispersed by drawing up and expelling several times using 6 mL syringe and an 18G needle. Cell suspension and additional wash from the petri dish

were filtered (100 μ m) and washed. Harvested spleen was processed by mechanical disruption between two microscope glass slides in a petri dish with PBS. Cell suspension was obtained following filtration (100 μ m) and RBC lysis using RBC Lysis Buffer (eBioscience) and PBS wash. Fluorescently labeled cells were analyzed and presence of CFSE and Violet dye were reported at % WT or %Sdc-1^{-/-} macrophages respectively.

4.5.4 Statistical Analysis

Means and SEM were calculated for each parameter. All data were analyzed via 2-tailed Student *t* test. Values of $P < 0.05$ were considered statistically significant.

CHAPTER 5

EFFEROCYTOSIS AS A REGULATOR OF MACROPHAGE CXCR4 EXPRESSION AND EGRESS IN INFLAMMATION

Some key features that characterize the resolution of inflammation are the effective clearance of apoptotic cells and the successful emigration of macrophages out of the inflammatory site to the draining lymphatic network. While the mechanisms by which monocytes are recruited into the inflammatory site is well understood, not as much is known regarding the regulation of macrophage motility within the inflammatory site itself and how macrophages are cleared at the end of inflammatory response. Given macrophage prevalence in chronic inflammatory disease and the importance of its clearance in order to achieve resolution, it is important to understand the mechanisms by which macrophage motility can be regulated during inflammatory responses.

5.1 Introduction

Similar to other immune cells, macrophage migration is governed through the expression of chemokines and chemokine receptors. However, unlike other immune cells, chemokine receptor expression pattern on macrophages is not as well described and understood. Recent studies that have highlighted the complexities of macrophage polarization states have served as an indication that the regulation of macrophage chemokine receptors is likely to be just as complex. Just as each macrophage polarization state can be associated with its own unique biochemical signature and functional activity,

it is highly likely that each polarization state is also associated with distinct motility behavior and unique chemokine receptor expression profile.

We, and others, have noted that the intrinsic motility of M1 macrophages is greatly reduced when compared to their M2 counterparts. The work done by Vereyken et al has demonstrated that classically activated M1 and alternatively activated M2a macrophages display distinct adhesion capacity and migratory behavior toward neuronal conditioned medium [11]. They reported M1 and M2a macrophages migrate distinctly toward conditioned media obtained from cultured central nervous system cells, with M2a macrophages displaying a significantly higher motility response. M2a macrophages were also reported to display lower adhesion to extracellular matrix compared to M1 macrophages. This was in agreement with previous studies where macrophage exposure to M1 stimulus resulted in increased expression of adhesion receptors [225, 226]. Overall, the M2a phenotype was associated with macrophages that displayed lower adhesion and higher motility, which the authors postulated made the macrophages more prone to migrate toward neurons during inflammation, where they can exert their anti-inflammatory and tissue healing effect.

Despite these marked differences in motility potential, chemokine receptor expression pattern in relation to macrophage polarization state has not been thoroughly investigated. CCR7 has previously been suggested as a candidate chemokine receptor mediating macrophage clearance out of inflamed tissue [198, 199]. In an animal model of atherosclerosis in which plaque regression and macrophage clearance was observed following transplantation of plaque containing arterial segments from ApoE^{-/-} to wild type mice, in vivo blockade of CCR7 function by injection of antibodies that bind to CCR7

ligands, resulted in the abrogation of pro resolution responses that were previously observed [198]. However, in a more recent study that was performed using an atherosclerosis model system that did not involve surgery, in which atherosclerosis was reversed with ApoE encoding adenoviral vectors, CCR7 was found not to be involved in the lowering of macrophage content within the atherosclerosis plaque [41]. In fact, macrophage CCR7 expression has actually been link to the more sessile, proinflammatory M1 subtype [129, 200]. This warrants a more systemic examination of motility potential and chemokine receptor expression in M2 macrophages and how they are regulated during inflammation.

In this chapter, we demonstrate that M1 and M2 macrophages are associated with distinct chemokine receptor expression. We report that M2 macrophages display high expression level of CXCR4, CX3CR1 and CCR10 compared to M1 macrophages. The expression level of CCR7 was found to be higher in M1 macrophages, as previously reported [129, 200]. Notably, efferocytosis, a process that has been characterized as the physiological pro resolution switch for macrophages, was found to upregulate CXCR4 expression in both human and murine macrophages in vitro. Enhanced expression of CXCR4 during murine model of peritonitis was found to participate in macrophage clearance from the peritoneal cavity to the draining lymphatic system. In addition, macrophages expressing high level of CXCR4 were also found to express higher level of Sdc-1 on their surface. Thus, our data suggest that efferocytosis driven CXCR4 expression can participate in the regulation of macrophage motility within inflammation site and potentially affect the dynamic and outcome of many inflammatory diseases.

5.2 Results

5.2.1 Characterization of chemokine receptor expression in M1 and M2 macrophages

As discussed in the previous chapter, our characterizations of Sdc-1⁺ macrophages have indicated that these macrophages display characteristics that are associated with M2 polarization. In order to perform a more systemic examination of chemokine receptor expression in polarized macrophages, the gene expression profile of chemokine receptors in M2/Sdc-1⁺ and M1 macrophages were investigated using the chemokines and receptors RT2 profiler PCR array. RNA was extracted from M1 macrophages and M2/Sdc-1⁺ macrophages, which were obtained following macrophage exposure to LPS and IFN- γ , or macrophage exposure to adenosine and enrichment using the MACS system, respectively. Analysis of the chemokine receptor expression levels in these macrophages, which were normalized to GAPDH expression level, revealed that CXCR4, CX3CR1 and CCR10 were expressed at a significantly higher level in M2/Sdc-1⁺ compared to M1 macrophages (Table 5.1). We utilized a transwell chemotaxis experimental setup to determine if these chemokine receptors were expressed on macrophage surface and if they were functionally active. In addition, the effect of macrophage polarization on chemotaxis response was also examined. In this experiment, 1×10^5 of macrophages were added to the upper chamber transwell plates with 5 μ m pore size following exposure to M1 or M2 stimulus, while corresponding ligands were added to the bottom chamber. Using this setup, we found that M2/Sdc-1⁺ macrophages displayed higher chemotaxis response toward each of the corresponding ligands compared to M1 macrophages (Fig. 5.1). This agrees with our previous finding where M2 macrophages were found to display higher intrinsic motility compared to M1

macrophages. We have also previously demonstrated that the absence of Sdc-1 expression was associated with reduced motility (Chapter 3). Thus, the experiment was repeated using Sdc-1^{-/-} macrophages to investigate Sdc-1 involvement in macrophage chemotaxis. As expected, significantly reduced chemotaxis response was observed in the absence of Sdc-1 expression when macrophages were exposed to adenosine (Fig 5.2A), while low chemotaxis response was observed in both groups when macrophages were exposed to M1 stimulus (Fig. 5.2B).

Table 5.1 mRNA expression profile of chemokine receptors in Sdc-1⁺ compared to M1 macrophages. RNA was extracted from Sdc-1⁺ or M1 macrophages and analyzed on the chemokines and receptors RT1 profiler PCR array; expression were normalized to GAPDH and were reported as expression index. All data represent the mean (n=3).

Gene	Sdc-1 ⁺ :M1	Ligand	Gene Information
CXCR4	48	CXCL12/SDF-1	Co-receptor used by HIV virus to infect macrophages
CCR10	12	CCL27	Participate in the regulation of skin inflammation
CX3CR1	12	CX3CL1	Participate in the recruitment of monocyte into the inflammatory site
CCR7	-31	CCL19, CCL21	Participate in lymph node homing and is also a marker for proinflammatory M1 macrophages

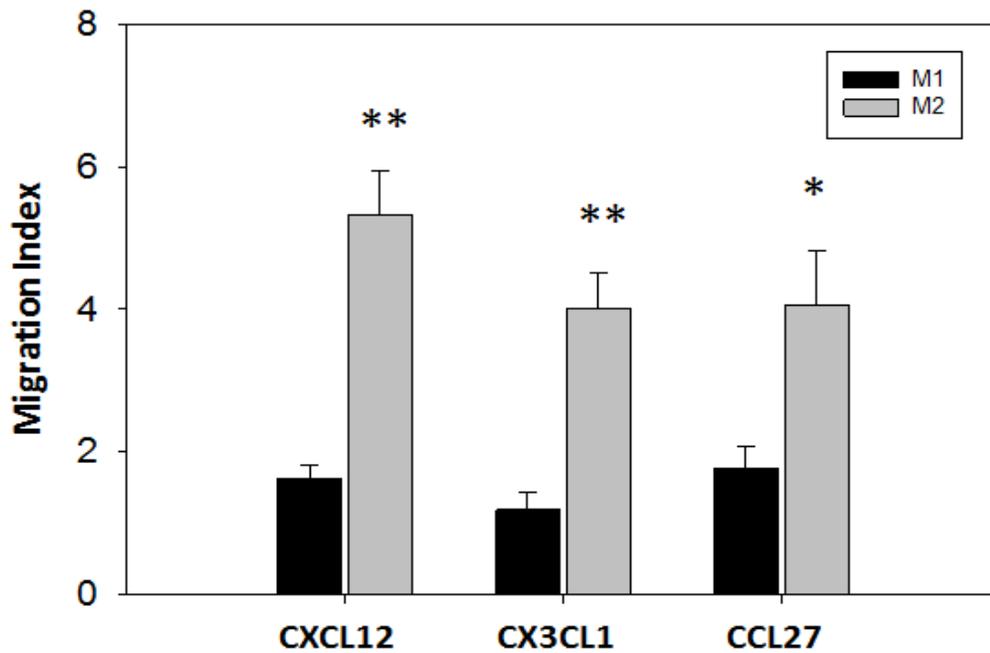


Figure 5.1. Chemotaxis response of M1 and Sdc-1⁺ macrophages. M1 or Sdc-1⁺ macrophages were placed in the upper chamber of a transwell while select ligands were placed in the lower chamber (CXCL12, 200ng/mL; CX3CL1, 400ng/mL; CCL27, 250ng/mL). After 18h, macrophages that have migrated to the lower surface of the chamber were stained using hematoxylin, counted and reported as migration index (n=4). *P<0.05. **P<0.01. All data represent the mean \pm SEM.

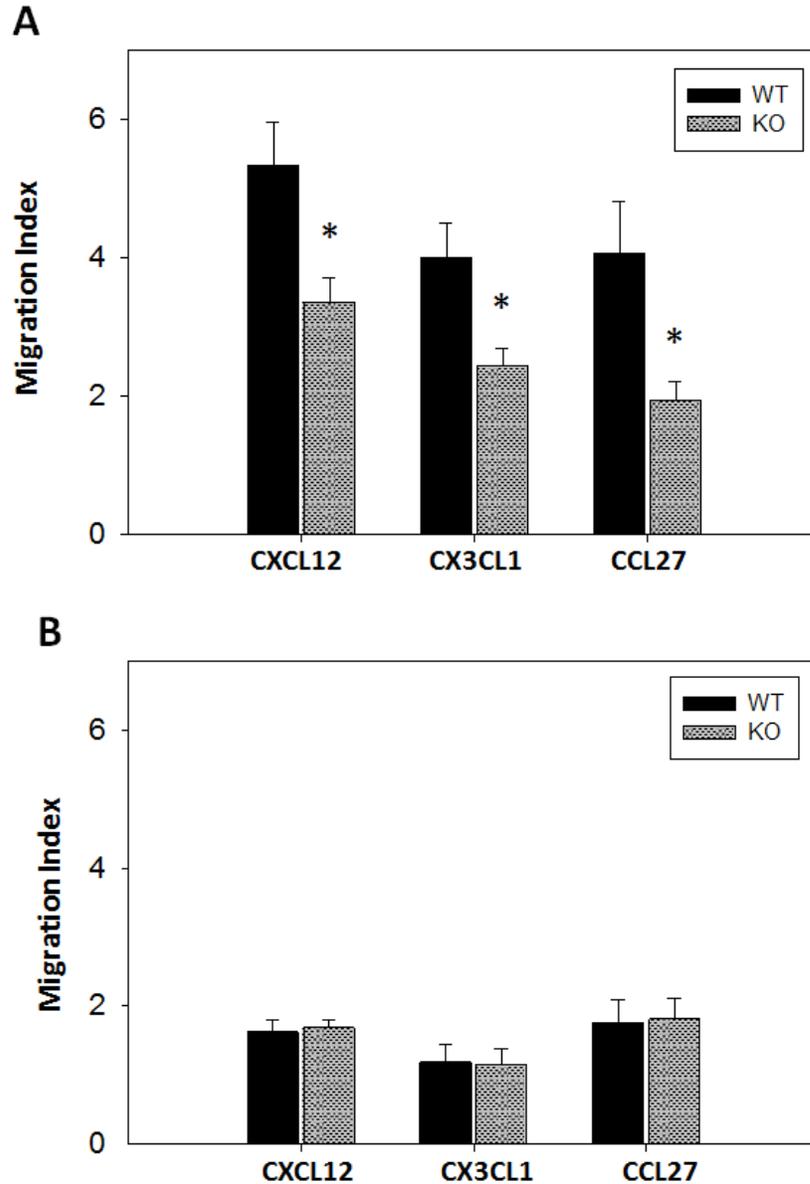


Figure 5.2. Absence of Syndecan-1 expression is associated with reduced chemotaxis response. **A-B.** *Sdc-1*^{+/+} (WT) and *Sdc-1*^{-/-} (KO) macrophages were placed in the upper chamber of a transwell while select ligands were placed in the lower chamber (CXCL12, 200ng/mL; CX3CL1, 400ng/mL; CCL27, 250ng/mL). After 18h, macrophages that have migrated to the lower surface of the chamber were stained using hematoxylin, counted and reported as migration index. **A.** Chemotaxis response of macrophages under M1 polarization was analyzed (n=4). *P<0.05. **B.** Chemotaxis response of macrophages under Ado treatment was analyzed (n=4). All data represent the mean ± SEM.

5.2.2 Efferocytosis as a regulator of macrophage chemokine receptor expression

Our characterizations of M1 and M2/Sdc-1⁺ macrophages have suggested that macrophage polarization can affect both macrophage motility and chemotaxis response. In particular, certain chemokine receptors including CXCR4, CX3CR1 and CCR10 were upregulated only on M2/Sdc-1⁺, but not M1 polarized macrophages. While these receptors can be expressed in vitro following macrophage treatment with adenosine, how their expression is regulated in vivo is unclear. As we have previously demonstrated, efferocytosis can regulate the expression of macrophage Sdc-1. Thus, we elected to investigate the potential of efferocytosis in regulating macrophage chemokine receptor expression. We investigated the ability of apoptotic macrophages to stimulate macrophage migration toward CXCL12, CX3CL1 and CCL27 using a standard Transwell migration assay. We found that apoptotic cells exposure did stimulate macrophage migration toward CXCL12 (Fig.5.3A). However, we did not observe an impact on CX3CL1 and CCL27 driven chemotaxis (Fig.5.3A). This suggested that efferocytosis could stimulate macrophage CXCR4 expression, but not CX3CR1 or CCR10. In order to confirm the effect of efferocytosis on macrophage CXCR4 expression, flow cytometry analysis was performed on macrophages that have been exposed to apoptotic cells. Flow cytometry analysis confirmed that macrophage CXCR4 expression was indeed upregulated in response to efferocytosis (Fig.5.3B). This finding indicates that both Sdc-1 and CXCR4 expression can be upregulated in response to efferocytosis. To determine the correlation between macrophage CXCR4 expression and Sdc-1 expression, macrophages that have been exposed to apoptotic cells were separated into two populations based on their CXCR4 expression level: low (CXCR4^{lo}) or high

(CXCR4^{hi}) expression level (Fig.5.4A). Flow cytometry was then utilized to further characterize the Sdc-1 expression of these populations. As expected, high CXCR4 expression was associated with high Sdc-1 expression (Fig.5.4B).

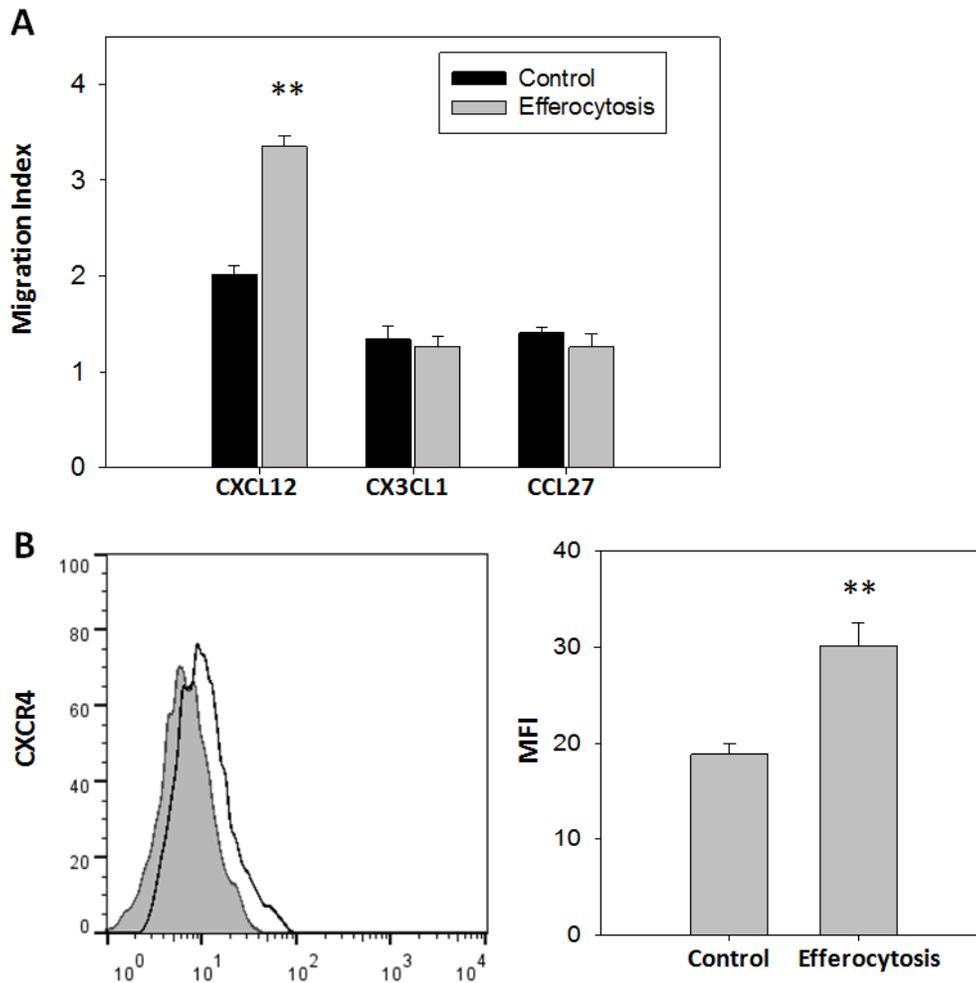


Figure 5.3. Efferocytosis induces macrophage CXCR4 expression. **A.** Macrophage chemotaxis toward CXCL12, a ligand of CXCR4, can be induced following exposure to apoptotic cells. Macrophage chemotaxis response was analyzed using transwell migration assay. Macrophages were placed in the upper chamber of a transwell while select ligands were placed in the lower chamber (CXCL12, 200ng/mL; CX3CL1, 400ng/mL; CCL27, 250ng/mL). Apoptotic macrophages were also added to the upper chamber of the experimental group. Macrophages that have migrated to the lower surface of the chamber that have been exposed to apoptotic cells were stained using hematoxylin, counted and reported as migration index (n=4). **P<0.01. **B.** Macrophage CXCR4 expression can be induced following exposure to apoptotic cells. Surface expression of CXCR4 was analyzed on macrophages that have been exposed to apoptotic cells using flow cytometry and quantified as MFI (filled: control, black line: experimental; n=4). **P<0.01. All data represent the mean \pm SEM.

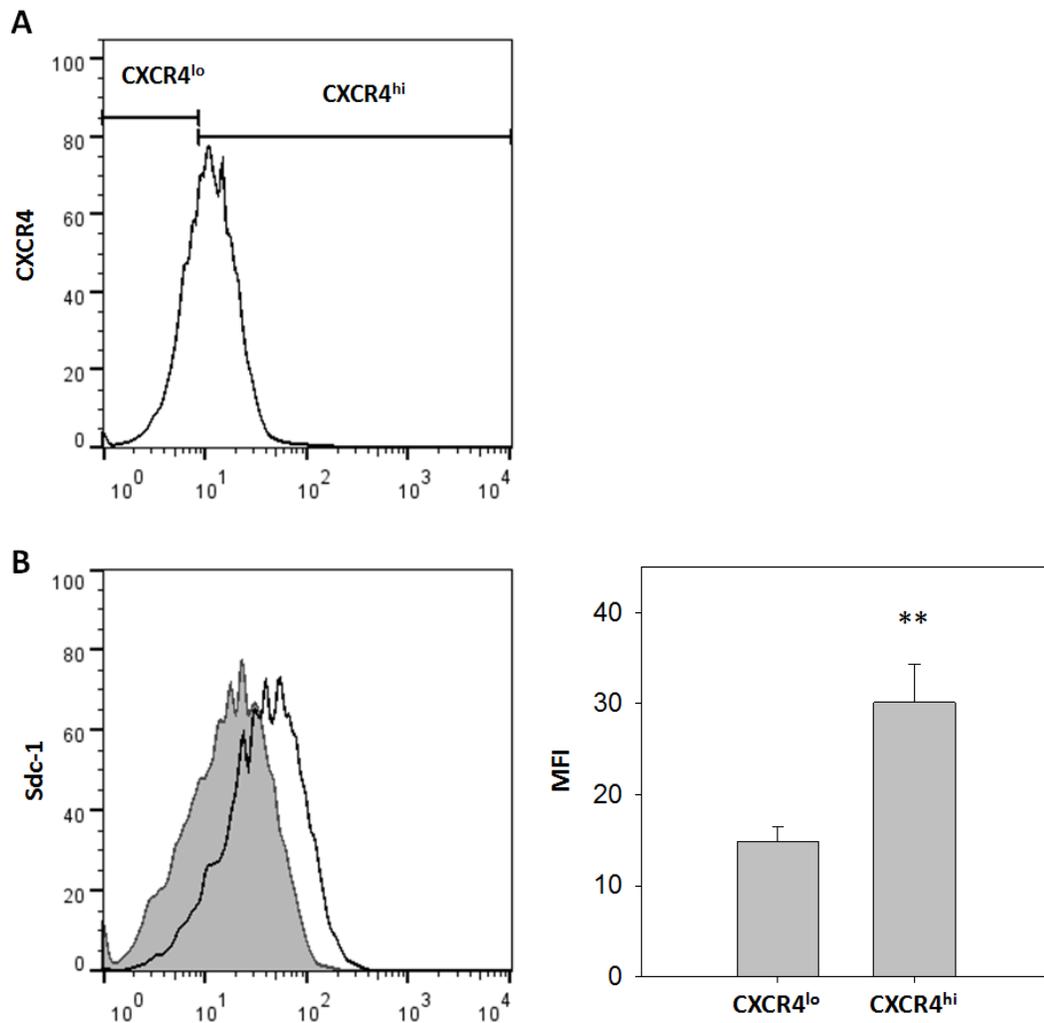


Figure 5.4. High CXCR4 expression level in response to efferocytosis is associated with high Sdc-1 expression. **A.** Macrophages that have been exposed to apoptotic cells were separated into two population based on their CXCR4 expression level: low expression level (CXCR4^{lo}) and high expression level (CXCR4^{hi}). **B.** Sdc-1 expression was analyzed in CXCR4^{lo} and CXCR4^{hi} macrophage population using flow cytometry and quantified as MFI (filled: CXCR4^{lo}, black line: CXCR4^{hi}, n=4). **P<0.01. All data represent the mean ± SEM.

5.2.3 Involvement of CXCR4 in mouse peritonitis model

Macrophage efferocytosis and clearance out of the inflamed tissue are hallmark of tissue undergoing inflammation resolution. However, the chemokine receptors that are potentially involved in macrophage clearance out of the inflamed tissue are still unknown. Given our finding that macrophage CXCR4 expression was upregulated following macrophage exposure to apoptotic cells, we elected to investigate the potential involvement of CXCR4 in macrophage motility during inflammation using a murine model of peritonitis, an acute inflammatory model that has been widely utilized to study leukocyte recruitment and motility during inflammation. Early during peritonitis, neutrophils are recruited into the peritoneal cavity where they subsequently turn apoptotic and are cleared by the macrophages through efferocytosis. Thus, macrophages were collected early during the peritonitis response and analyzed using flow cytometry to check for their CXCR4 expression. Of note, macrophages collected from mice not undergoing peritonitis were also collected for analysis, and no inherent CXCR4 expression was observed (Fig.5.5A). Significantly, we observed an upregulation in macrophage CXCR4 expression at 18 hours post peritonitis induction, indicating that CXCR4 was potentially involved in the regulation of macrophage migration during inflammation (Fig 5.5A). To determine if CXCR4 was actively involved in macrophage migration during peritonitis, the effect of CXCR4 blockade was analyzed using AMD3100. AMD3100, also known as Plerixafor, is a compound that works as an antagonist to CXCR4 [228-230]. While the inhibitory effect of AMD3100 on CXCR4 mediated chemotaxis on hematopoietic stem cells and tumor cells has been well studied [228-230], the effect of AMD3100 on CXCR4 mediated macrophage chemotaxis has not

been previously characterized. Thus, the ability of AMD3100 to block macrophage migration toward CXCL12 was first analyzed using a standard Transwell migration assay, where it was found to effectively limit macrophage chemotaxis toward CXCL12 (Fig.5.5B). To determine CXCR4 involvement in vivo, mice undergoing peritonitis were injected with AMD3100 or PBS at 18 hours post peritonitis injection. Flow cytometry analysis performed on peritoneal exudates obtained 6 hours post injection showed that macrophage count was increased in mice that were injected with AMD3100 compared to mice that received PBS injection (Fig.5.5C). This result indicates that CXCR4 is involved in regulating macrophage migration during inflammation in peritonitis model and that its blockade can result in macrophage retention at the site of inflammation.

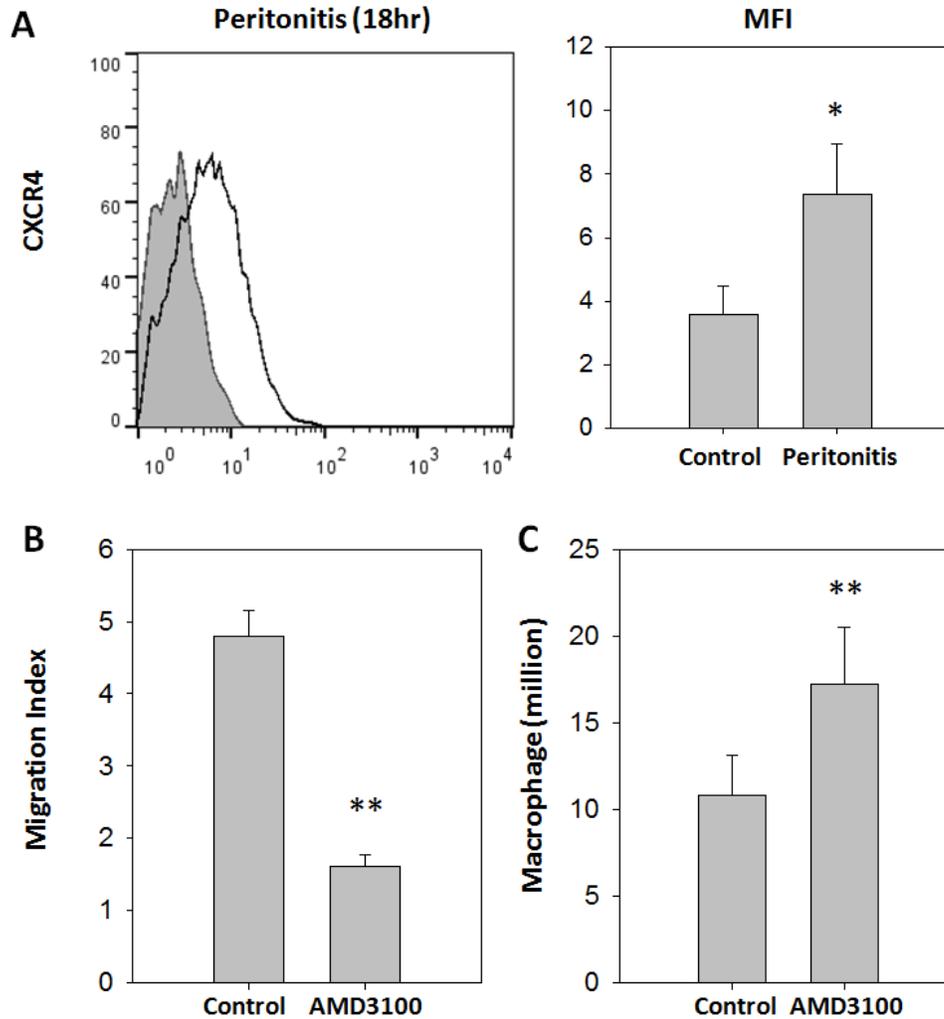


Figure 5.5. Macrophage CXCR4 is involved in the regulation of macrophage migration during peritonitis. **A.** Macrophages were obtained 18 hours post peritonitis induction and CXCR4 expression was analyzed using flow cytometry and quantified as MFI (filled: control, black line: experimental; n=3). *P< 0.05. **B.** Effect of AMD3100 on macrophage chemotaxis toward CXCL12 was investigated by adding AMD3100 to the upper well in the experimental group (25µg/mL); no treatment was added to the control group. After 18 hours, macrophages that migrated to the lower surface of the membrane were stained, counted and reported as migration index (n=3). **P<0.01. **C.** Effect of AMD3100 on macrophages during peritonitis response was investigated by AMD3100 injection (125 µg/mouse) at 18 hr post induction. Following 6 hours incubation period, peritoneal macrophages were obtained, stained and counted (n=6-8). **P<0.01. All data represent the mean ± SEM.

5.2.4 CXCR4 involvement in macrophage egress to the lymphatics

Macrophage accumulation in chronic inflammation is thought to be the result of macrophage egress failure during inflammatory response. Previous studies have indicated that macrophage clearance during inflammation resolution involved macrophages egress to the surrounding draining lymph nodes [3, 41, 51, 128, 231]. Our observation that AMD3100 treatment resulted in macrophage accumulation within the peritoneal cavity suggested the involvement of CXCR4 in macrophage egress out of the inflammatory site to the draining lymph nodes. To test this hypothesis, we utilized an adoptive transfer model in which fluorescently labeled macrophages were transferred into the peritoneal cavity of mice undergoing the same stage of peritonitis. In order to verify CXCR4 involvement in macrophage clearance to the draining lymph nodes, macrophages were first treated with AMD3100 prior to the adoptive transfer process and compared to untreated control macrophages. 10 hours of post-adoptive transfer, the number of fluorescently labeled macrophages in the peritoneal cavity was reduced in both experimental and control group. However, a significantly greater amount of labeled macrophages were found in the peritoneal cavity of mice that were treated with AMD3100 (Fig.5.6A). This result suggests that macrophage accumulation in the peritoneal cavity following AMD3100 administration was the result of macrophage failure to egress from the inflammation site. In order to track the migration of the fluorescently labeled macrophages, mesenteric lymph nodes were harvested from both control and experimental groups. Fluorescently labeled population recovered from the lymph nodes and spleen was analyzed using flow cytometry analysis and counted using counting beads. As expected, the numbers of labeled macrophages that was recovered

from the mesenteric lymph nodes was significantly lower in mice that were treated with AMD3100 compared to the control group(Fig.5.6B). Given our previous finding of macrophage retention in the peritoneal cavity following AMD3100 treatment, the observation of reduced labeled macrophages in the draining lymph nodes was not surprising. Overall, these results suggest that CXCR4 is important in regulating macrophage egress from the inflammation site to the draining lymphatics and is potentially critical in the resolution of inflammatory response.

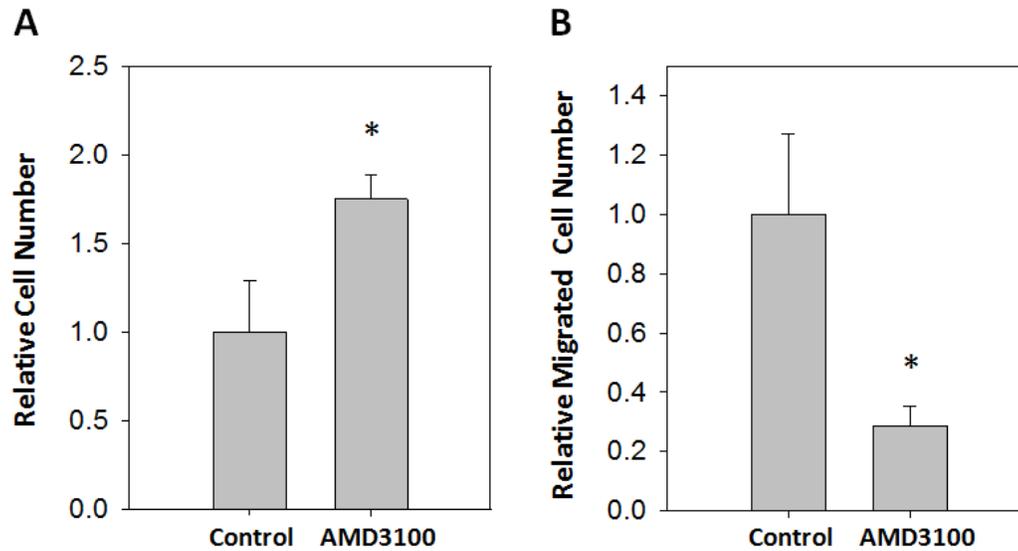


Figure 5.6. CXCR4 blockade by AMD3100 resulted in delayed macrophage clearance to the lymphatics. Fluorescently labeled macrophages were adoptively transferred into the peritoneal cavity of WT mice undergoing the same stage of peritonitis. Mice were sacrificed at 10 hours post adoptive transfer and the peritoneal lavage and mesenteric lymph nodes were harvested and processed. **A.** Peritoneal lavage was analyzed for fluorescently labeled macrophages that remained in the peritoneal cavity (n=6). *P<0.05. **B.** Cell suspension obtained from the lymph node were analyzed to determine the presence of fluorescently labeled macrophages (n=10-11). *P<0.05. All data represent the mean \pm SEM.

5.2.5 CXCR4 is expressed on human macrophages under M2 polarization.

Thus far our results suggest that CXCR4 is a chemokine receptor that is expressed on M2 macrophages and is also potentially involved in macrophage egress during resolution. To provide further insight into the regulation of CXCR4 expression on polarized macrophages, flow cytometry was used to analyze chemokine receptor expression on polarized human macrophages. Human macrophages were obtained from circulating blood monocytes that were cultured in the presence of macrophage colony stimulating factor (M-CSF). M1 polarization was achieved using IFN- γ and LPS treatment, while M2 polarization was achieved using adenosine treatment. Surface expression of HLA-DR and CD163, known markers for M1 and M2 polarization respectively, and CXCR4 was analyzed. As expected, flow cytometry analysis of polarized human macrophages demonstrated significantly higher level of HLA-DR expression on M1 macrophages compared to M2 macrophages (Fig.5.7A). On the contrary, M1 macrophages were found to display significantly lower level of CD163 compared to M2 macrophages (Fig.5.7A). Significantly, we observed that CXCR4 expression, while nonexistent in M1 macrophages, was drastically upregulated on M2 macrophages (Fig.5.7A). Similar to murine macrophages, we found that exposure of macrophages to apoptotic cells resulted in the upregulation of CXCR4 expression (Fig.5.7B). Thus, in line with our finding in murine system, CXCR4 is also associated with M2 polarization state in human macrophages.

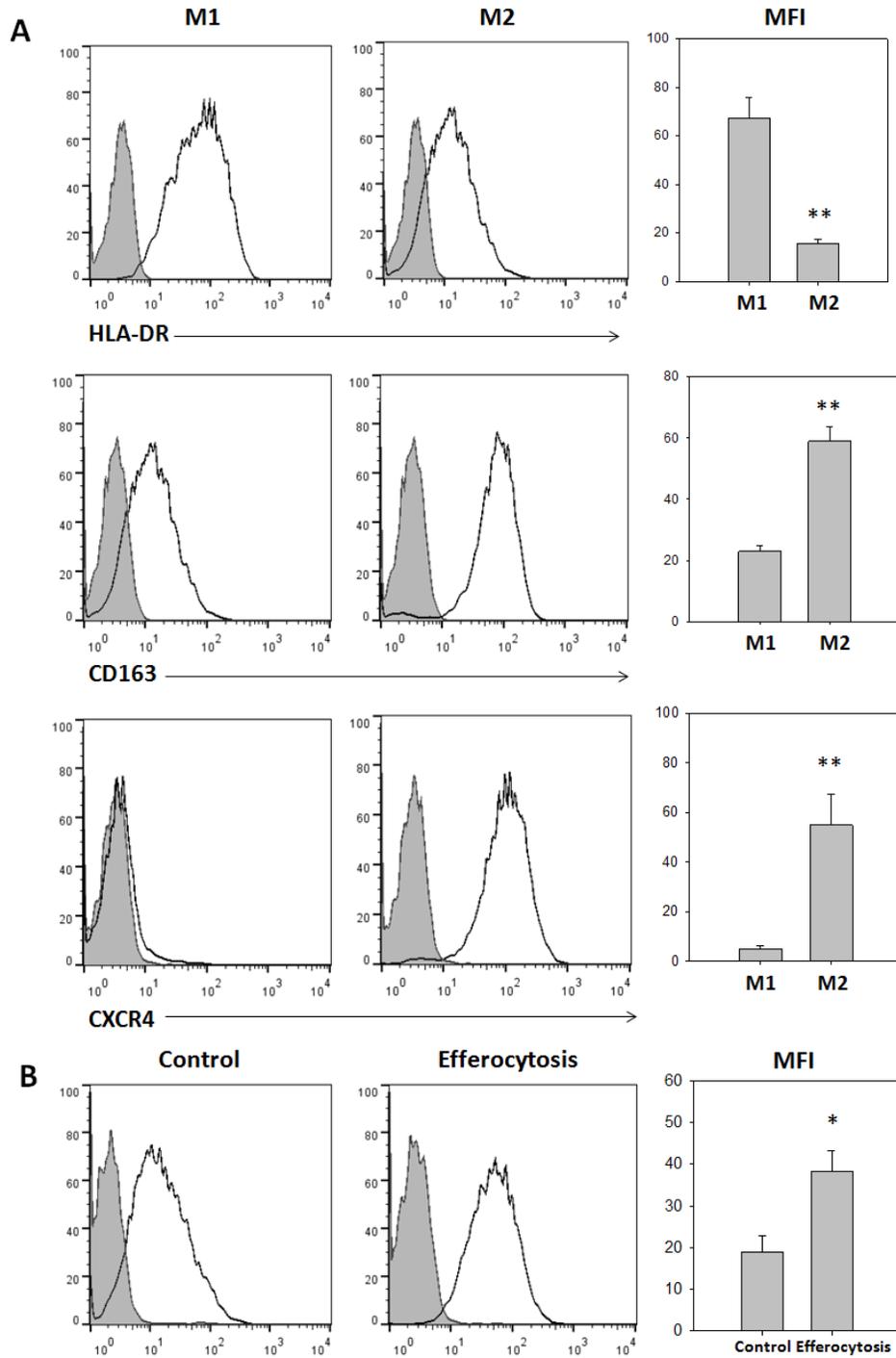


Figure 5.7. Sdc-1 expression is associated with M2 polarization state and CXCR4 expression on human macrophages. **A.** The expression of HLA-DR and Sdc-1 was analyzed on human macrophages following exposure to M1 stimulus (IFN- γ , 20ng/mL + LPS, 100ng/mL), or 6-benz-cAMP (100 μ M), (n=3-4). **P<0.01. **B.** Macrophage CXCR4 expression following efferocytosis was analyzed using flow cytometry and quantified as MFI (n=3). *P<0.05. All data represent the mean \pm SEM.

5.3 Discussion

Macrophage accumulation within the inflammation site is one of the defining features of many chronic inflammatory disease, including rheumatoid arthritis, chronic liver disease, chronic kidney disease and atherosclerosis [2, 121, 126, 154, 232]. While previous efforts have been focused on understanding monocyte recruitment into the inflammation site and their roles in the orchestration of inflammatory response, recent findings have suggested that both efferocytosis and macrophage egress can also have a profound effect on the outcome of inflammation. In fact, efferocytosis has often been suggested to dampen macrophage activation state and trigger macrophage egress from the inflammation site into the draining lymph nodes [50, 99, 103, 171]. Despite these assertions, the link between macrophage efferocytosis and macrophage motility is not actually well understood. In this project, our investigation have identified CXCR4 as an endogenous receptor that was expressed on M2 polarized macrophages. Notably, macrophage CXCR4 expression was found to coincide with Sdc-1 expression, which we have demonstrated to associate with M2 polarization. Moreover, we provide evidence that macrophage CXCR4 expression can be triggered by efferocytosis. Significantly, CXCR4 was also found to participate in macrophage egress from inflammation site to the draining lymph nodes. Thus, macrophage expressed CXCR4 is potentially a part of the mechanism by which efferocytosis results in macrophage egress from the inflammation site, leading to the resolution of inflammatory response.

Macrophage involvement during the course of inflammatory response is well established. The concept of macrophage polarization states, inflammatory mediator secretion and efferocytosis and how they may affect inflammation have been described in

numerous independent studies [10, 13, 99, 103, 124, 140, 171, 202]. Although macrophage is known to orchestrate inflammatory response, the notion that macrophage motility can have a major impact on inflammatory outcome has not been widely considered before. However, several reports have demonstrated that macrophage motility can potentially play a crucial role in determining the outcome of inflammatory response [29, 203]. A study done by van Gils et al has demonstrated that blockade of macrophage migration could result in macrophage accumulation within the vascular wall, which resulted in the pathogenic progression of atherosclerosis [203]. Conversely, stimulating macrophage migration within the plaque was associated with increased macrophage clearance and plaque regression. Enhanced macrophage motility has also been linked with the anti-inflammatory cAMP signaling and M2 polarization state [29].

Unfortunately, characterization of both macrophage polarization states and specific chemokine receptors expressions were not performed in those studies. On the contrary, our findings have clearly demonstrated that M1 and M2 macrophages differ in their chemokine receptor expression. In particular, CXCR4, CX3CR1 and CCR10 were found to be significantly upregulated in M2/Sdc-1⁺ compared to M1 macrophages. In agreement with previous reports, CCR7 was found to be upregulated in M1 compared to M2/Sdc-1⁺ macrophages [129, 200]. Not surprisingly, the unique chemokine receptor repertoire of polarized macrophages resulted in distinct chemotaxis behavior; M1 polarization significantly inhibited, while M2 polarization promoted macrophage chemotaxis toward CXCL12, CX3CL1 and CCL27. Thus, macrophage motility is emerging as an important mechanism through which macrophages orchestrate the inflammatory response.

Although macrophage exposure to adenosine can result in the expression of CXCR4, CX3CR1 and CCR10 on M2/Sdc-1⁺ polarized macrophages, the biophysical process by which their expression can be regulated in vivo is unknown. Efferocytosis is a process that has long been recognized to be necessary in the resolution of inflammation. In addition to ensuring the prompt removal of cellular debris and apoptotic cells out of the inflamed tissue, efferocytosis has also been suggested to promote resolution by promoting macrophage egress [49, 99]. A recent study by Driscoll et al has also demonstrated that enhanced macrophage efferocytosis could accelerate macrophage clearance from the peritoneum in a murine peritonitis model, thus promoting inflammation resolution [49]. In addition, efferocytosis has been reported to result in the release of adenosine during inflammation [219]. Given the link between efferocytosis, enhanced motility and adenosine release, efferocytosis appears to be a likely candidate for triggering CXCR4, CX3CR1 and CCR10 expression in vivo. Unexpectedly, we did not observe a significant impact on macrophage chemotaxis toward CX3CL1 and CCL27 following exposure to apoptotic cells. However, macrophage chemotaxis response toward CXCL12 was found to be significantly upregulated following efferocytosis, indicating an upregulation of CXCR4 expression, which was confirmed through flow cytometry analysis. Significantly, high macrophage CXCR4 expression level was associated with high Sdc-1 expression level. Overall, our finding indicates that Sdc-1 and CXCR4 can be expressed on macrophages under similar physiologic condition during inflammation response.

CXCR4 involvement in the regulation of macrophage motility in vivo was analyzed using peritonitis, a commonly utilized acute inflammatory model for studying

leukocyte recruitment and migration. Neutrophils, which are recruited early during peritonitis response, can quickly turn apoptotic following their extravasation and are subsequently cleared through efferocytosis. We demonstrated that macrophages that were obtained during this time frame expressed CXCR4 on their surface. Notably, we observed no evidence of CXCR4 expression on macrophages obtained from mice not undergoing peritonitis. CXCR4 involvement in the regulation of macrophage migration during inflammation was verified using AMD3100, a CXCR4 antagonist that blocks macrophage chemotaxis toward CXCL12 in vitro. Consistent with our hypothesis, CXCR4 blockade via AMD3100 treatment resulted in macrophage accumulation within the peritoneal cavity, which was presumably caused by reduced macrophage emigration. Overall, our results suggest that although M2 macrophages express CXCR4, CX3CR1 and CCR10, only CXCR4 expression is regulated through efferocytosis during inflammation. In addition, CXCR4 expression is potentially a part of the mechanism activated by efferocytosis that is designed to support macrophage egress from the inflammation site.

Macrophage accumulation during chronic inflammation can be partly explained by reduced macrophage clearance out of the inflammatory site. While the fate of emigrating macrophages is not fully known, macrophage presence in the draining lymphatics during resolution has been documented previously [99, 128, 198, 199]. Given the therapeutic potential of stimulating macrophage egress, uncovering mechanisms that regulate macrophage clearance toward the draining lymph nodes has been the subject of various reports [49, 99, 128, 198, 199]. Schiff-Zuck et al reported that enhanced efferocytosis was associated with macrophage clearance toward the lymph nodes during

the resolution of murine peritonitis [99]. Cao et al has also demonstrated the importance of macrophage integrin Mac-1 in accelerated macrophage efflux from the peritoneal cavity to the lymphatics in response to LPS stimulation during peritonitis [128]. Consistent with these reports, we found that macrophage clearance from the inflamed peritoneal cavity involved macrophage migration toward the lymphatics. Utilizing an adoptive transfer model of fluorescently labeled macrophages, we demonstrated the involvement of CXCR4 in macrophage egress toward lymph nodes. Significantly, CXCR4 blockade resulted in the accumulation of fluorescently labeled macrophages in the peritoneal cavity, which was coupled with reduced clearance to the draining lymph nodes. These results suggest that CXCR4 is one of the chemokine receptors that is involved in driving macrophage egress during inflammation resolution. To our knowledge, this is the first report in which macrophage expressed CXCR4 has been demonstrated to actively participate in macrophage egress toward the lymphatics during inflammatory resolution.

5.4 Conclusion

Several reports have established the importance of macrophage egress on the resolution of inflammatory response. We therein demonstrate that polarized macrophages express a unique chemokine receptor repertoire and chemotaxis response. While CXCR4, CX3CR1 and CCR10 were all upregulated on adenosine induced macrophages, only CXCR4 expression was regulated through macrophage efferocytosis. Notably, macrophage CXCR4 was found to participate in macrophage egress from the inflammation site toward the draining lymphatics. Thus, CXCR4 expression on macrophages may play an important role not only in regulating macrophage migration

response during inflammation but also in potentially influencing the outcome of many inflammatory diseases.

5.5 Methods

5.5.1 Macrophage Culture and Activation

C57BL6 wild type (WT) mice were purchased from Jackson Laboratory. Syndecan-1 (Sdc-1)^{-/-} mice (back crossed 10 times onto a C57BL6 background) was kindly provided by Dr. Pyong Woo Park, (Baylor College of Medicine, Houston, TX). All procedures were performed in male mice according to a protocol approved by Emory University and Beth Israel Deaconess Medical Center Institutional Animal Care and Use Committee.

Murine peritonitis was induced by the IP injection of 0.5mL of 6% sterile TG broth, which was prepared from dehydrated TG medium (Sigma Aldrich) and sterile water. Peritoneal macrophages were obtained through lavage using 5mL of sterile PBS with 1mM EDTA at 4 or 5 days following peritonitis initiation, unless otherwise noted. Peritoneal macrophages were cultured in DMEM (Cellgro) containing 10% FBS (Cellgro), 100U/mL penicillin and 100µg/ml streptomycin sulfate (Sigma Aldrich) at 37°C in 5% CO₂ humidified incubator. Where indicated, macrophage M1 activation was induced by culturing macrophages overnight in the presence of Ifn-γ (100U/mL) and LPS (100ng/mL). Sdc-1 expression was induced by culturing macrophage overnight with Adenosine (Sigma Aldrich, 375 µM).

Human acute monocytic leukemia cell line was obtained from ATCC and cultured as previously described (Chapter 3, Method Section). Monocytic differentiation was induced by incubation of 1x10⁷ cells in 100x200mm tissue culture dish in culture media that has been supplemented with 50ng/mL of phorbol 12-myristate 13-acetate (PMA) (Sigma Aldrich) for 3 days. After the differentiation period, M1 polarization was

induced by culturing macrophages overnight with Ifn- γ (20ng/mL) and LPS (100ng/mL), while M2 polarization was induced by culturing macrophage overnight with Ado (375 μ M).

5.5.2 PCR Array

mRNA expression analysis of 84 genes that encode chemokines and their receptors were performed using mouse chemokines and receptors RT2 profiler PCR array (SABiosciences). RNA was extracted from classically activated M1 and Sdc-1⁺ (Adenosine) macrophages with TRIzol reagent (Invitrogen) and purified using RNeasy mini kit (Qiagen) according to manufacturer's protocol. A total of 1 μ g of RNA from each sample were converted to cDNA using the RT² first strand kits (SABiosciences) and was then subjected to PCR array analysis on the Applied Biosystems 7900 using the RT² qPCR Master Mixes according to manufacturer's protocol (SABiosciences). Data were analyzed using the web-base PCR array data analysis tool from SABiosciences. mRNA expression level for each gene was normalized to the expression level of glyceraldehydes-3-phosphate dehydrogenase (GAPDH) using the equation $2^{-(Ct[\text{gene of interest}] - Ct[\text{GAPDH}])}$, where Ct is the threshold cycle. Normalized gene expression levels of experimental samples (Sdc-1⁺ macrophages) were obtained by comparing the gene expression level to control samples (M1 macrophages). Each reported value represents the mean fold increase or fold decrease of mRNA expression in experimental samples relative to controls.

5.5.3 Flow cytometry

Isolated macrophages for surface marker expression analysis were passed through a 70-micron cell strainer to remove debris and then counted and resuspended in FACS

buffer (1% BSA in PBS) at a concentration of 5×10^6 /mL. Staining was performed by combining the manufacturer's recommended quantity of primary antibody to 100 μ L of cell suspension, followed by 30 minutes of incubation at 4°C. Cells were washed twice before being analyzed on the flow cytometer (LSRII, Becton Dickinson).

The cell antibodies used mouse cell staining included: anti-F4/80 (BM8), anti-CD138/Sdc-1 (281-2) and anti-CD184/CXCR4 (2B11); negative controls with isotype IgG were included for each marker. Anti-F4/80 antibody was obtained from eBioscience and the rest were obtained from BD Biosciences.

The cell antibodies used for human cell surface markers analysis included: anti-HLA-DR (G46-6), anti-CD163 (GHI/61), and anti-CD184/CXCR4 (12G5); negative controls with isotype IgG were included for each marker. All of the antibodies were obtained from BD Biosciences.

5.5.4 Generation of Apoptotic Macrophages and Efferocytosis Experiment

Apoptotic peritoneal macrophages and RAW 264.7 macrophages were generated as described previously (Chapter 3, Method Section). Apoptotic THP1 macrophages were generated similarly by exposure of plated macrophages to UV light. However, macrophages were exposed to UV light for 10 minutes and were additionally cultured for 2.5 hours

To investigate murine macrophage CXCR4 expressing following efferocytosis, apoptotic RAW 264.7 cells were added to peritoneal macrophages (15 to 1 ratio) cultured in DMDM supplemented with 0.5% FBS, which were then placed at 37°C in 5% CO₂ humidified incubator. Following 2 hours of incubation, excess apoptotic cells were removed by washing with PBS and remaining macrophages were cultured for

additional 40 hours. Macrophages were removed using harvested using non-enzymatic cell dissociation buffer (Sigma Aldrich) and washed using PBS prior to being stained for flow cytometry analysis.

To investigate human CXCR4 expression following efferocytosis, apoptotic THP1 cells were added to peritoneal macrophages (15 to 1 ratio) cultured in RPMI supplemented with 0.5% FBS, which were then placed at 37°C in 5% CO₂ humidified incubator. Following 2 hours of incubation, excess apoptotic cells were removed by washing with PBS and remaining macrophages were cultured for additional 40 hours. Macrophages were removed using harvested using non-enzymatic cell dissociation buffer (Sigma Aldrich) and washed using PBS prior to being stained for flow cytometry analysis.

5.5.5 Macrophage Chemotaxis

Chemotaxis response of polarized macrophages was analyzed by loading 100 µL of peritoneal macrophages from Sdc-1^{+/+} or Sdc-1^{-/-} mice that have been resuspended at a concentration of 1x10⁶/mL in DMEM containing 0.5% FBS, onto the upper well of a 24-well transwell permeable supports with a pore size of 8µm (Corning); DMEM containing 0.5% FBS was added onto the lower well. During the 4-hour-incubation period, M1 stimulus or Adenosine was added onto both upper and lower chambers. After 4h, the media and stimulus was removed and fresh DMEM containing 0.5% FBS added onto both chambers. The chemokines CXCL12 (200ng/mL), CX3CL1 (400ng/mL) or CCL27 (250ng/mL) was added onto the lower chambers of experimental group, while no chemokines were added in the control groups. After 18h of incubation, macrophages that have migrated onto the lower well surface of the membrane were fixed with 10%

formalin in PBS for 10 minutes. Macrophages that adhere to the upper well were scraped and the membrane was then stained with hematoxylin. For each replicate, 3 separate wells containing peritoneal macrophages obtained from separate mouse were included. The numbers of macrophages in three independent fields of view were counted in each well and averaged. Migration was quantified as migration index by dividing the number of macrophages counted in experimental compared to control group.

To investigate the chemotaxis response of macrophages that have been exposed to apoptotic cells, 100 μ L of peritoneal macrophages that have been resuspended at a concentration of 1×10^6 /mL in DMEM containing 0.5% FBS were added onto the upper chamber of a transwell permeable support; 600 μ L of DMEM containing 0.5% FBS was also added onto the lower chamber. Macrophages were allowed to adhere for 1 h before 5×10^5 apoptotic peritoneal macrophages in 50 μ L of media was added onto the upper well of experimental group; 50 μ L of media was added onto the upper well of control group. After 4 hours of incubation, the base media was removed from the lower chamber, and media containing the chemokines CXCL12 (200ng/mL), CX3CL1 (400ng/mL) or CCL27 (250ng/mL) was added onto the lower chambers of both experimental and control group. After 18 hours, migration response was analyzed as described above.

5.5.6 CXCR4 blocking – AMD3100

In order to investigate effect of AMD3100 on macrophage chemotaxis toward CXCL12 in vitro, chemotaxis experiment was set up as described above using macrophages that have been treated with Ado. AMD3100 (Sigma Aldrich) was added onto the upper well of the experimental group (25 μ g/mL); no treatment was added to the

control group. After 18 hours, macrophages that migrated to the lower surface of the membrane were stained, counted and reported as migration index as described above.

To investigate the effect of AMD3100 on macrophage migration in vivo, Mice were IP injected with 0.5 mL of 6% sterile TG broth to induce peritonitis. AMD3100 (200 μ L of 625 μ g/mL solution in PBS) was then IP injected at 18 hours post TG injection, while equal volume of sterile PBS was injected in the control group. Mice were sacrificed after 6 hours, and peritoneal lavage was performed using 5mL of sterile PBS with 1mM EDTA. The number of macrophages in the peritoneal lavage was determined by performing total cell count and analyzing the percentages of macrophages using flow cytometry analysis with mouse F4/80 antibody staining.

5.5.7 Adoptive transfer

For adoptive cell transfer experiments, peritonitis was induced in both donor and recipient mice. Macrophages were obtained through lavage at 18 hours post peritonitis induction and labeled with 3 μ M CellTace Violet (Invitrogen) according to manufacturer's suggestion. Labeled macrophages were then counted and resuspended in sterile PBS. In the experimental group, AMD3100 was then added at a concentration of 25 μ g/mL and was incubated at 4°C in the dark for 30 minutes; no treatment was added onto the control group. Cell suspension was then injected into the peritoneal cavity of recipient mice undergoing the same stage of peritonitis (18 hours post peritonitis induction). Recipient mice also received AMD3100 injection intraperitoneally in the experimental group (200 μ L of 625 μ g/mL solution in PBS), while equal volume of sterile PBS was injected in the control group. Recipient mice were then sacrificed 10 hours post adoptive transfer and the mesenteric lymph nodes were harvested and processed for flow

cytometry analysis, as described previously (Chapter 3, Method Section). Relative cell number was obtained by dividing the number of recovered cells with the average of the recovered cells from control mice.

5.5.8 Statistical Analysis

Means and SEM were calculated for each parameter. All data were analyzed via 2-tailed Student *t* test. For the PCR array analysis, the P values were calculated based on a Student *t* test of the replicate $2^{-(Ct[\text{gene of interest}] - Ct[\text{GAPDH}])}$ values for each gene in both the control and experimental groups. Values of $P < 0.05$ were considered statistically significant.

CHAPTER 6

CONCLUSION

This last decade has witnessed a profound leap in our understanding of inflammation resolution. Once regarded as a passive process, resolution is now understood to be an active process that is regulated by numerous complex mechanisms that work together to ensure the return of tissue homeostasis. Ongoing research has discovered numerous chemical mediators, signaling pathways and biophysical processes that are essential in the resolution of inflammation. Despite the significant progress that has been made, many of the mechanisms involved in inflammation resolution are still poorly understood. Given the prevalence of chronic inflammatory diseases and the lack of effective therapeutics, developing a deeper understanding of the mechanisms involved in the resolution of inflammation can help lead to novel treatment approaches.

In this dissertation, we uncovered a unique population of macrophages expressing Sdc-1 that are present within the vascular wall of mice undergoing atherosclerosis. Consistent with previous publications by our group and others that report the protective effect of Sdc-1 expression in various inflammation models, the presence of Sdc-1 expressing macrophage was found to limit atherosclerosis progression. In addition, macrophage Sdc-1 expression was associated with anti-inflammatory M2 polarization state, efferocytosis, high intrinsic motility and macrophage egress from the inflammation site to the lymphatic network, all of which are considered essential for the successful resolution of inflammation.

In the first phase of this study, we attempted to characterize the polarization state of macrophages that are expressing Sdc-1 in vitro and within atherosclerotic plaque. We investigated the expression of several surface markers that are known to be expressed on M1 and M2a macrophages. We also tested the iNOS activity, arginase activity, IL-10 production, phagocytosis, intrinsic motility and adhesion characteristic of Sdc-1 expressing macrophages against standard polarized M1, M2a and M2c macrophage populations. Sdc-1^{-/-} macrophages were also utilized in select studies to confirm Sdc-1 involvement in the regulation of macrophage functions. These characterization efforts established the anti-inflammatory nature of Sdc-1 expressing macrophages, in conjunction with increased macrophage migration response. Importantly, efferocytosis was found to be a possible regulator of macrophage Sdc-1 expression. The correlation that we discovered between efferocytosis, Sdc-1 expression and enhanced motility provided motivation to investigate the effect of macrophage Sdc-1 expression on macrophage motility in vivo.

Based on the results obtained from our in vitro characterization of Sdc-1 expressing macrophages, we hypothesized that the absence of Sdc-1 expression on macrophages can result in delayed macrophage clearance from the inflammation site to the draining lymphatics network. Macrophage egress to the lymphatics is understood to be necessary for successful resolution of inflammation. Our study utilizing Sdc-1^{+/+} and Sdc-1^{-/-} mice and a TG induced peritonitis model supported our hypothesis. While the neutrophil response was found to be similar between the two groups, the absence of Sdc-1 was found to result in the accumulation of macrophages in the peritoneal cavity, which is associated with delayed macrophage clearance. In addition, we confirmed that the

delayed macrophage clearance was associated with dampened macrophage egress toward the draining lymph nodes. Significantly, these findings demonstrated a direct involvement of Sdc-1 in macrophage egress during inflammatory responses, which supports the notion that Sdc-1 expression is a part of the mechanisms that regulate the resolution of inflammatory response.

Lastly, we attempted to identify specific chemokine receptors involved in macrophage egress during the resolution of inflammation. We identified 3 chemokine receptors that were upregulated in Sdc-1 expressing M2 polarized macrophages, CX3CR1, CXCR4 and CCR10. However, only CXCR4 was found to be expressed and functionally active following macrophage exposure to apoptotic cells. Similar to macrophage Sdc-1 expression, we were able to confirm that efferocytosis can also result in the upregulation of macrophage CXCR4 expression. In fact, macrophages expressing high level of CXCR4 were found to also express high level Sdc-1 expression on their surface. This observation suggests that Sdc-1 and CXCR4 are expressed on macrophage surface under similar physiological stimulus. Further tests utilizing AMD3100, a CXCR4 blocker, in murine peritonitis model confirmed that macrophage CXCR4 is involved in the regulation of macrophage clearance. CXCR4 blockade resulted in the accumulation of macrophages within the peritoneal cavity which was associated with delayed macrophage egress toward the draining lymph nodes. This agrees with our previous finding, which linked the absence of Sdc-1 expression with delayed macrophage egress during peritonitis. Overall, we were able to demonstrate that macrophage Sdc-1 expression is associated with CXCR4 expression, and are both important in ensuring macrophage clearance during inflammatory resolution.

In summary, we demonstrate that Sdc-1 expression on macrophages is associated with an anti-inflammatory, pro resolution polarization state and enhanced migration. Macrophage Sdc-1 expression was also found to be correlated with CXCR4 expression, both of which can be upregulated in response to efferocytosis. Importantly, both macrophage Sdc-1 and CXCR4 was found to participate in the regulation of macrophage egress from the inflammation site to the draining lymphatics during the resolution of inflammatory response. While the overall mechanisms regulating resolution processes are unknown, our work has managed to identify two components that are involved in the process: macrophage Sdc-1 and CXCR4. Collectively, these results reinforce the physiological significance of macrophage efferocytosis and macrophage motility as endogenous modulators of the inflammatory response.

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