 Syndecan-1 Modulates the Motility and Resolution Responses of Macrophages

Julianty Angsana, Jiaxuan Chen, Sumona Smith, Jiantao Xiao, Jing Wen, Liying Liu, Carolyn A. Haller, Elliot L. Chaikof

Objective—Syndecan-1 (Sdc-1) is a member of a family of cell surface proteoglycans, which 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. In this study, we report the functional significance of Sdc-1 in polarized macrophage populations and its role in adhesion and motility events relevant to resolution of the inflammatory program.

Approach and Results—Macrophage Sdc-1 expression is associated with differentiated M2 macrophages with high intrinsic motility, and Sdc-1 deficiency is characterized by impaired migration and enhanced adhesion. Leukocyte infiltration and emigration were examined in a thioglycollate-induced model of peritonitis in Sdc-1<sup>−/−</sup> and Sdc-1<sup>+/−</sup> mice. Although the infiltration of inflammatory cells was similar in both cohorts, a significant delay in the lymphatic clearance of Sdc-1<sup>−/−</sup> macrophages was observed. Moreover, we observed enhanced inflammation and greater burden of atherosclerotic plaques in ApoE<sup>−/−</sup>Sdc-1<sup>−/−</sup> mice maintained on a Western diet.

Conclusions—These results demonstrate that defective motility in Sdc-1<sup>−/−</sup> macrophages promotes a persistent inflammatory state with relevance to the pathogenesis of atherosclerosis. (Arterioscler Thromb Vasc Biol. 2015;35:332-340. DOI: 10.1161/ATVBAHA.114.304720.)

Key Words: cell movement ▪ macrophage ▪ syndecan-1

A persistent population of activated lesional macrophages has been linked to the progression of atherosclerosis. Unrestrained macrophage activation plays a vital role in pathogenic remodeling and plaque destabilization that can culminate in rupture and acute coronary syndromes. Intensive research efforts are focused on understanding the regulatory mechanisms that signal resolution programs characteristic of a healthy inflammatory response and a return to tissue macrophage homeostasis. Nonphlogistic clearance of apoptotic cells has been suggested as a key event that drives macrophage resolution via anti-inflammatory signaling and enhanced egress of macrophages from inflammatory foci. Indeed, secondary necrosis observed in advanced atherosclerotic plaques may be a signature of failed resolution programs in sessile lesional macrophages that do not efficiently clear apoptotic debris and likewise do not propagate signals to silence inflammation and exit the vascular wall. Such observations have renewed interest to understand the macrophage motility dynamics, as they relate to a persistent inflammatory state within the vessel wall with the hope of developing new strategies to limit macrophage burden in atherosclerosis.

Plaque regression has been documented in numerous animal studies and remains the ultimate therapeutic goal in the treatment of human atherosclerosis. Recent investigations using a mouse surgical transplant model of plaque regression have suggested that dramatic improvements in plasma lipoprotein profiles can induce monocyte-derived cells to emigrate out of the inflamed vascular wall and that this process correlates with reduced plaque burden. Cell tracking studies identified egress depots in the iliac and hepatic lymph nodes, suggesting dissemination through draining lymph nodes, as well as reentry into the blood stream. Consistent with this hypothesis of failed macrophage clearance in chronically inflamed vascular lesions, Moore et al have reported the expression of inhibitory guidance cues, netrin-1, and semaphorin 3E, in atheroma macrophages. Moore et al offered a paradigm of competing trafficking signals to explain the macrophage retention, where neuronal guidance molecules secreted by activated macrophages may serve as a stop signal to inhibit the activation of the actin cytoskeleton and override the chemokine-mediated macrophage emigration from the atherosclerotic wall. Conversely, data have been reported in an apoE complementation model of regression, where reduced macrophage burden is strongly correlated with decreased monocyte recruitment and apoptotic turnover rather than migratory egress. Collectively, these reports emphasize the complexity in dissecting the macrophage resolution response and provide
motivation for continued investigations of inflammatory effectors that govern macrophage retention in atherosclerosis.

In this study, we report the functional significance of the cell-surface heparan sulfate proteoglycan, syndecan-1 (Sdc-1), on macrophage adhesion and motility events relevant to inflammatory resolution. We have previously reported macrophage Sdc-1 as an endogenous modulator of the inflammatory response in abdominal aortic aneurysm, and we speculated that Sdc-1-expressing macrophages represented an alternatively activated regulatory population in the chronically inflamed wall. Anti-inflammatory functions of Sdc-1 are often attributed to the sequestration of inflammatory mediators by pendant glycosaminoglycans; however, Sdc-1 has also been reported to regulate cell adhesion and migration. Macrophage Sdc-1 protein expression is governed through the cAMP/protein kinase A signaling cascade, which is a signaling program reported to inhibit inflammation in macrophages. However, there have been no previous reports examining Sdc-1 expression as a function of macrophage polarization. Moreover, predicting the anticipated role of Sdc-1 in macrophage motility is not intuitive because Sdc-1 has been reported to both inhibit and augment motility in cell- and tissue-dependent contexts. We demonstrate that Sdc-1 expression is specific to differentiated M2 macrophages with high intrinsic motility and that Sdc-1 deficiency is characterized by impaired migration and enhanced extracellular matrix adhesion. In a self-resolving model of inflammation, we examined both infiltration and emigration of macrophages in wild-type (Sdc-1+/+) and Sdc-1-deficient (Sdc-1−/−) mice. Significantly, we observed no difference in the kinetics of leukocyte influx or the magnitude of emigration of macrophages in wild-type (Sdc-1+/+) and Sdc-1-deficient (Sdc-1−/−) mice. Macrophage Sdc-1 expression is part of a broad program to regulate the trafficking of alternatively activated macrophages.

**Methods**

Materials and Methods are available in the online-only Data Supplement.

**Results**

**Sdc-1 Expression on Polarized Macrophage Populations**

Macrophage activation has been operationally divided into 2 subsets: classically activated proinflammatory M1 and alternatively activated M2. M2 polarization encompasses a broad spectrum of macrophage activation, and additional subdivisions have been suggested to clarify functional phenotypes, such as M2a to distinguish traditional Th2-type (IL-4 and IL-13) polarization and M2c to distinguish anti-inflammatory (IL-10, TGF-β, and glucocorticoid) polarization. Cell-surface marker expression, biochemical signature, and phagocytic capacity of Sdc-1 positive macrophages were characterized for M1, M2a, and M2c standard populations generated from elicited peritoneal macrophages. Flow cytometry was used to measure the cell-surface expression of MHCII, CD86, and programmed death ligand 2 (PD-L2), 3 markers that have been previously reported to associate with M1 or M2 polarization. M1-polarized cells displayed characteristically high expression of the antigen presenting molecule, MHCII, and the coactivating molecule, CD86 (Figure 1A). M2a-polarized cells were most easily characterized as MHCII+ with high expression of the PD-L2, a Th2-stimulated molecule that inhibits T-cell proliferation. M2c does not positively correlate with MHCII, CD86, or PD-L2 consistent with reports that IL-10 deactivates macrophages. Sdc-1 expressing macrophages, which can be enriched after treatment with a membrane-permeant cAMP analogue or adenosine-induced activation of endogenous adenylyl cyclase (Figure 1B; media control presented in Figure I in the online-only Data Supplement), displayed the strongest similarity to M2c with MHCIIlo, CD86lo, and PD-L2− signatures.

Macrophage populations were further assayed for soluble mediators, with elevated nitrite levels observed in the M1 population (Figure 1C), whereas arginase activity was enhanced in M2a macrophages (Figure 1D). A significant increase in IL-10 production was observed for Sdc-1-expressing macrophages when compared with M1- or M2a-polarized populations. However, M2c-polarized macrophages secreted the highest level of IL-10 (Figure 1E). Finally, bead phagocytosis, which has been reported to be upregulated in alternatively activated M2 macrophages, was found to be significantly elevated in all populations when compared with M1 (Figure 1F). Characterization of Sdc-1 expression in polarized standard populations suggests that Sdc-1 protein expression is not induced within the context of a primary Th1 (IFN-γ) or Th2 (IL-4) inflammatory response (Figure 1G) but instead may be displayed in those macrophages that have received a deactivation response, typified by decreased antigen presentation (MHCIIlo) and increased immunosuppression (IL-10+). Induction of Sdc-1 was not observed with IL-10 treatment (Figure 1G); however, additional protocols commonly reported to suppress or deactivate macrophages were examined, including exposure to TGFβ or glucocorticoids, as well as effector cytokine-mediated deactivation. Significantly, upregulation of Sdc-1 protein and mRNA was observed after effector cytokis of apoptotic macrophages (Figure 1H and 1I; Figure II in the online-only Data Supplement), suggesting a physiological program for expression that is consistent with deactivation and inflammatory resolution.

**Nonstandard Abbreviations and Acronyms**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>ApoE</td>
<td>apolipoprotein E</td>
</tr>
<tr>
<td>PD-L2</td>
<td>programmed death ligand 2</td>
</tr>
<tr>
<td>Sdc-1</td>
<td>syndecan-1</td>
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Figure 1. Sdc-1 expression on polarized macrophage populations. A, Sdc-1-expressing macrophages were examined against standard populations for surface expression of M1 (MHCII and CD86) and M2a (PD-L2) markers, and data are displayed in histogram format against the IgG control (gray) and graphed as mean fluorescent intensity (MFI). B, Sdc-1 expression can be induced on F4/80 positive macrophages after exposure to 6-bnz-cAMP or adenosine. C, Elevated M1-induced inducible nitric oxide synthase activity was characterized via Griess reaction. D, Arginase activity was measured in cell lysates and reported as fold increase over unstimulated control macrophages. E, IL-10 was measured via conditioned media ELISA. F, Percent phagocytosis was characterized in polarized populations by fluorescent bead uptake. G, Sdc-1 expression is not induced under standard M1, M2a, or M2c conditions. H and I, Sdc-1 expression is induced after efferocytosis of apoptotic macrophages. H, Data plotted as histogram (Sdc-1, white) against IgG control (gray) and as MFI. I, Quantitative polymerase chain reaction analysis of Sdc-1 mRNA in Sdc-1 wild-type and knockout macrophages +/- efferocytosis. Data are representative of triplicate sample means±SEM, *P<0.05, and **P<0.01.
Leukocyte Chemoattractive Potential of Sdc-1 Macrophages

In vitro phenotypic analysis suggests that macrophage Sdc-1 expression is not consistent with a classically activated M1 subtype, and subsequent quantitative real-time reverse transcription polymerase chain reaction studies confirmed this distinction. A higher level of proinflammatory genes and chemokines was observed in M1 versus Sdc-1-enriched macrophages (Tables I and II). Given the effect that leukocyte recruitment has on inflammatory outcome, we examined the chemoattractive potential of conditioned media generated from M1 or Sdc-1-enriched populations toward purified circulating monocytes or activated CD4 T-cells. Significantly, conditioned media from Sdc-1 macrophages elicited significantly reduced chemotaxis of monocytes and CD4 T-cells (Figure 2A and 2B; controls presented in Figure III in the online-only Data Supplement). These results suggest that although M1 macrophages actively contribute to the progression of inflammation, Sdc-1-expressing macrophages should correlate with reduced leukocyte influx in vivo.

Distinct Motility Behavior in Polarized Macrophage Populations

Decreased migratory capability may contribute to macrophage retention in chronic inflammatory environments. Given that Sdc-1 has been traditionally studied in the context of cell adhesion and migration, we examined the functional consequence of macrophage Sdc-1 deficiency on motility and adhesion in polarized populations. Motility was first investigated by characterizing the migration of Sdc-1+/+ and Sdc-1−/− macrophages toward fetal bovine serum using a Boyden chamber assay. Cells were specifically induced toward an M1 or Sdc-1+ phenotype. We observed dramatically dampened motility in classically activated Sdc-1+/+ and Sdc-1−/− M1 macrophages, with no significant difference noted between genotypes (Figure 3A). However, when the migration of Sdc-1+/+ and Sdc-1−/− macrophages was examined under conditions to induce a Sdc-1+ phenotype through the addition of adenosine to basal media, we observed a significant reduction in efferocytosis events for Sdc-1−/− when compared with Sdc-1+/+ macrophages (Figure 3C). When efferocytosis efficiency is examined under static conditions, we observed no difference between Sdc-1+/+ and Sdc-1−/− macrophages, which is consistent with our hypothesis that defective motility in Sdc-1−/− macrophages may promote a persistent inflammatory state.

The adhesion profiles of Sdc-1+/+ and Sdc-1−/− macrophages were investigated in comparison with wild-type M1-induced macrophages. M1-induced macrophages displayed the greatest adhesion, with increased adhesion noted for Sdc-1−/− when compared with Sdc-1+/+ macrophages (Figure 3D). Subsequent staining of the actin cytoskeleton revealed impaired ability of Sdc-1−/− macrophages to form cell protrusions (Figure 3E and F), consistent with previous reports that Sdc-1 can regulate cell protrusions that may be functionally important in migration by mediating outward extension of cell edges.35,36 We confirmed that Sdc-1−/− macrophages polarize to the expected M1 or M2 phenotype when

Table 1. mRNA Expression Profile of Proinflammatory Genes in M1 vs Sdc-1−

<table>
<thead>
<tr>
<th>Gene</th>
<th>M1 vs Sdc-1−</th>
<th>Gene Information</th>
</tr>
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<tbody>
<tr>
<td>M-CSF</td>
<td>7</td>
<td>Monocyte/macrophage differentiation factor</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>29</td>
<td>Granulocyte differentiation factor</td>
</tr>
<tr>
<td>IL1α</td>
<td>14</td>
<td>Proinflammatory cytokine</td>
</tr>
<tr>
<td>NFκβ</td>
<td>6</td>
<td>Transcription factor for many inflammatory genes</td>
</tr>
<tr>
<td>MyD88</td>
<td>5</td>
<td>Adapter protein that participates in the activation of NFκβ</td>
</tr>
<tr>
<td>TNFα</td>
<td>103</td>
<td>Proinflammatory cytokine</td>
</tr>
</tbody>
</table>

*Genes that displayed at least a 5 fold difference in expression with P≤0.05 are reported.

Table 2. mRNA Chemokine Expression Profile in M1 vs Sdc-1−

<table>
<thead>
<tr>
<th>Gene</th>
<th>M1 vs Sdc-1−</th>
<th>Gene Information</th>
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<tbody>
<tr>
<td>CCL2/MCP1</td>
<td>78</td>
<td>Monocyte chemoattractant</td>
</tr>
<tr>
<td>CCL4/MIP1β</td>
<td>60</td>
<td>Induce neutrophil superoxide production</td>
</tr>
<tr>
<td>CCL5/RANTES</td>
<td>354</td>
<td>TNFα and IL1β inducible, T-cell chemoattractant</td>
</tr>
<tr>
<td>CL7/MCP-3</td>
<td>100</td>
<td>Monocyte chemoattractant</td>
</tr>
<tr>
<td>CCL8/MCP-2</td>
<td>27</td>
<td>Monocyte, T-cell and NK-cell chemoattractant</td>
</tr>
<tr>
<td>CCL12/MCP-5</td>
<td>834</td>
<td>Monocyte chemoattractant</td>
</tr>
<tr>
<td>CXCL1</td>
<td>32</td>
<td>Neutrophil and monocyte recruitment</td>
</tr>
<tr>
<td>CXCL2</td>
<td>67</td>
<td>Neutrophil recruitment</td>
</tr>
<tr>
<td>CXCL9</td>
<td>99768</td>
<td>IFNγ inducible, T-cell and monocyte recruitment</td>
</tr>
<tr>
<td>CXCL10</td>
<td>5019</td>
<td>IFNγ inducible, T-cell and NK-cell recruitment</td>
</tr>
<tr>
<td>CXCL11</td>
<td>211</td>
<td>IFNγ inducible, T-cell recruitment</td>
</tr>
</tbody>
</table>

*Genes that displayed at least a 5 fold difference in expression with P≤0.05 are reported.
incubated with Th1 or Th2 cytokines, thus the only phenotypic defect observed in Sdc-1−/− macrophages was reduced motility and enhanced adhesion (Figure VI in the online-only Data Supplement). Collectively, these results suggest that Sdc-1 expression modulates motility in mature-polarized macrophage populations and may limit macrophage retention in chronic inflammatory environments.

**Sdc-1 Contributes to the Resolution of Inflammatory Responses**

We used a thioglycollate-induced murine model of peritonitis as an example of self-resolving inflammation to investigate the role of Sdc-1 in leukocyte infiltration and resolution. Cell counts, neutrophil and macrophage populations, and Sdc-1 expression were recorded during a 12-day time frame. At the induction of peritonitis, Sdc-1 expression was not detected on peritoneal macrophages. However, 24 to 48 hours after onset of peritonitis, Sdc-1 macrophages were abundant and then decreased over the 12-day time course (Figure 4A). Analysis of total cell counts revealed no difference in the kinetics or magnitude of the initial inflammatory response between Sdc-1+/+ and Sdc-1−/− mice (Figure 4B, time, 0–2 days). Indeed, the evolution and resolution of the neutrophil response were identical in Sdc-1+/+ and Sdc-1−/− mice (Figure 4C). Significantly, the resolution of the late inflammatory response was delayed in Sdc-1−/− mice, with a greater number of macrophages detected at 8 to 12 days (Figure 4D). There was no difference in the magnitude or kinetics of macrophage influx between Sdc-1+/+ and Sdc-1−/− mice; however, the time interval required for a 50% to 25% reduction in the number of peritoneal macrophages was significantly prolonged in Sdc-1−/− mice (1.7 versus 4.7 days; \( P<0.05; \) Figure 4E; Figure VII in the online-only Data Supplement). This result is consistent with a delayed clearance of macrophages from the peritoneal cavity in the absence of Sdc-1 expression. Adoptive transfer was used to examine macrophage emigration into the lymphatic system during the resolution phase of peritonitis. Fluorescently labeled Sdc-1+/+ and Sdc-1−/− macrophages were introduced into the peritoneal cavity at day 3, and mesenteric lymph or spleen was harvested 18 hours post adoptive transfer. Significantly, an increase in lymphatic trafficking in Sdc-1+/+ macrophages was noted (Figure 3F). Thus, in vitro and in vivo migration studies suggest that Sdc-1 expression may be an important modulator of macrophage motility and, specifically,
may be an important functional component of macrophage emi-
gration and resolution.

Sdc-1 Limits Atherosclerotic Lesion Formation
To examine the role of Sdc-1 in atherosclerosis, ApoE−/−Sdc-1−/− mice were generated17 and the presentation of atherosclerosis was compared with that observed in ApoE−/− mice when both cohorts were maintained on a Western diet. At 8 weeks, brachiocephalic lesion area was quantified in consecutive tissue sections, and the inflammatory profile was examined using standard immunohistochemistry.45–48 We did not observe Sdc-1 staining in native brachiocephalic sections (data not shown), but by 8 weeks, Sdc-1 expression was clearly evident in macrophage positive areas around lesional plaques and within the adventitial tissue (Figure 5A and B; staining control in Figure VIII in the online-only Data Supplement). Although serum cholesterol levels were identical in both cohorts, a significant increase in atherosclerotic lesion area was measured in ApoE−/−Sdc-1−/− mice when compared with ApoE−/− mice (88.03±8.66 versus 57.40±8.08; P<0.05; Figure 5C; Figure IX in the online-only Data Supplement). Consistent with enhanced lesion formation, we observed enhanced expression of the macrophage M1 marker, inducible nitric oxide synthase, and greater necrotic area within Apoe−/−Sdc-1−/− lesions (Figure 5D and 5E).

To explore the relevance of our findings in human atheroscle-
rosis, we examined human carotid plaque sections for macro-
phage Sdc-1 staining. Atherosclerotic plaques were harvested during the course of carotid endarterectomy from symptomatic patients having reported a recent stroke or transient ischemic attack. Immunohistochemical staining revealed colocalization of macrophages and Sdc-1 positive staining within identical regions of the lesion (Figure 5F). To further investigate Sdc-1 expression in human macrophages, we examined polarization and motility in THP1 macrophages in vitro. Notably, Sdc-1 expression was consistent with M2 polarization and an enhanced motility profile (Figure X in the online-only Data Supplement).

Discussion
Recent findings suggest that the resolution of inflammation involves both efferocytosis and emigration of monocyte-derived cells out of sites of inflammation through nearby lymphatic vessels.1,49 However, both processes may be impaired in atherosclerosis, which accounts for macrophage accumulation within plaques.4,50 In this regard, the mechanism for impeded efferocytosis and emigration from plaques remains a topic of intense interest for potential therapeutic intervention. We have determined that macrophage Sdc-1 expression is associated with anti-inflammatory M2 polarization and enhanced motility in both murine and human macrophages. Sdc-1−/− macrophages displayed a significant reduction in motility and increased adhesion, and in vivo, Sdc-1 macrophage deficiency resulted in delayed egress of macrophages in a model of self-resolving peritonitis and enhanced atherosclerotic lesion formation in ApoE−/−Sdc-1−/− mice. These data highlight a functional role for Sdc-1 in the motility of alternatively activated macrophage populations and reinforce the significant relationship of the dynamics of macrophage motility to responses that underlie the resolution of inflammation.

In adult tissue, Sdc-1 expression is restricted to noncirculating plasma cells and epithelial cells. Expression can be induced in additional cell types, such as endothelial cells, smooth muscle cells, fibroblasts, and macrophages.17,18 There are limited studies documenting in vivo expression and functional relevance of Sdc-1 expression in macrophages. Indeed, Sdc-1 expression is not observed in circulating monocytes or resident macrophages; however, Sdc-1 mRNA is upregulated on macrophage...
elicitation.\textsuperscript{51} Protein expression is governed via cAMP/protein kinase A as a post-transcriptionally regulated event, which has also been reported in keratinocytes, mesenchymal cells, and epithelial cells.\textsuperscript{52–54} The molecular mechanisms governing this non-traditional regulation of expression have yet to be elucidated, but presumably, on-demand expression would allow macrophages to rapidly tailor cell surface properties to meet specific functional requirements. Agents that elevate intracellular cAMP, such as E-type prostaglandins, membrane permeable cAMP analogues, or adenosine, promote Sdc-1 protein expression in macrophages.

Nonphlogistic clearance of apoptotic cells, or efferocytosis, is a key process regulating macrophage M2 inflammatory polarization and resolution. Indeed, there are numerous reports detailing macrophage immune silencing as the consequence of efferocytosis. Macrophage engulfment of apoptotic cells can trigger the secretion of soluble anti-inflammatory mediators, such as IL-10, TGF-\(\beta\), adenosine, and prostaglandin E\(_2\).\textsuperscript{21,27,57} Moreover, efferocytosis has been suggested to modulate macrophage motility and promote emigration,\textsuperscript{1} and our studies confirm that efferocytosis induces Sdc-1 protein expression. Given that the only phenotypic defect in Sdc-1\textsuperscript{−/−} macrophages was reduced motility and enhanced adhesion, it is plausible that efferocytosis-induced Sdc-1 expression contributes to the emigration program of macrophages and resolution of inflammation.

Traditionally, Sdc-1 has been studied in the context of cell adhesion and migration in mesenchymal and epithelial cell types. Because a membrane-anchored proteoglycan was expressed on the basolateral surface of epithelial cells, Sdc-1 supports cell contact with extracellular matrix proteins and modulates cytoskeletal organization, leading to changes in cell shape and adhesion. Sdc-1 has been reported to augment epithelial migration in skin and cornea but inhibits migration in the lung by slowing focal adhesion disassembly.\textsuperscript{21,27,57} Both overexpression and gene knockout of Sdc-1 reduce migration in a wound-healing assay, suggesting that an optimal balance of surface expression is required to support the locomotion of mesenchymal and epithelial cells.\textsuperscript{28,58} There are limited reports documenting a functional role of Sdc-1 in adhesion or motility of leukocytes, which can use amoeboid migration without adherence to matrix components.\textsuperscript{59,60}
display intermediate migration speed and are capable of both amoeboid and mesenchymal migration. They do not exhibit large focal complexes consistent with their requirement for rapid migration responses. Sdc-1 has been linked to the formation of fascin-dependent cell protrusions, which can guide migration events. Fascin spikes, which have been reported in dendritic cells, are dynamic extensions of the plasma membrane that can extend and retract quickly and assemble in the absence of focal contacts. The morphological absence of cell protrusions is evident in Sdc-1−/− macrophages treated with agents that induce high cell surface Sdc-1 expression, such as membrane permeable cAMP analogues or adenosine. This morphological distinction coupled with a motility defect that is observed in the absence of Sdc-1 expression suggests that expression is critical in modulating cytoskeletal dynamics for this specific subset of M2 macrophages. Given the capacity of macrophages to engage in both integrin-mediated adhesion-dependent motility and adhesion-independent interstitial migration, future efforts will focus on examining the contribution of Sdc-1 within the context of both motility paradigms.

Recent investigations have highlighted the potential significance of heterogeneous populations of specialized macrophages in the initiation and resolution of inflammatory responses. One of the defining features of chronic inflammation is the persistence of activated macrophages. In atherosclerotic plaques, activated lesional macrophages possess a potent repertoire of proinflammatory mediators to sustain a sessile, regressive, but not progressive, plaques. Of monocyte-derived cells from atherosclerotic lesions characterizes the resolution by resolvins and glucocorticoids. Curr Opin Immunol. 2011;41:366–379.


There is intense interest in understanding why the macrophage population fails to resolve in atherosclerotic plaques, and recent findings suggest that macrophage migration out of the site of inflammation may be an important process to trigger the return to tissue homeostasis. Our data show that the cell-surface heparan sulfate proteoglycan, syndecan-1, modulates motility and adhesion in macrophage populations. In vivo, we observed that this motility defect translated to reduced macrophage trafficking during inflammatory resolution and enhanced atherosclerotic plaque formation. Collectively, these results support the notion that defective motility of mature macrophage populations may promote a persistent inflammatory state and suggest that syndecan-1 is part of a broad program to regulate resolution phase trafficking.

Significance

Atherosclerosis is a chronic inflammatory disease that is distinguished by the persistence of inflammatory macrophages in vascular lesions. There is intense interest in understanding why the macrophage population fails to resolve in atherosclerotic plaques, and recent findings suggest that macrophage migration out of the site of inflammation may be an important process to trigger the return to tissue homeostasis. Our data show that the cell-surface heparan sulfate proteoglycan, syndecan-1, modulates motility and adhesion in mature-polarized macrophage populations. In vivo, we observed that this motility defect translated to reduced macrophage trafficking during inflammatory resolution and enhanced atherosclerotic plaque formation. Collectively, these results support the notion that defective motility of mature macrophage populations may promote a persistent inflammatory state and suggest that syndecan-1 is part of a broad program to regulate resolution phase trafficking.
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SUPPLEMENTAL MATERIALS AND METHODS

Syndecan-1 Modulates the Motility and Resolution Responses of Macrophages

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METHODS

Mouse models. All experimental procedures were performed in male mice at maturity (8-12 weeks), according to protocol approval by Emory University and Beth Israel Deaconess Institutional Animal Care and Use Committees. C57BL/6J mice and ApoE−/− mice were purchased from Jackson Laboratory, Sdc-1−/− and ApoE−/−Sdc-1−/− were described previously. Western diet (Research Diets) was provided for eight weeks to induce atherosclerosis, N=19 ApoE−/− and N=19 ApoE−/−Sdc-1−/−. At harvest, mice were euthanized by CO2 and lesion area was quantified in the brachiocephalic artery with 3 consecutive tissue sections at a 50 µm interval distance using Image Pro software. Total serum cholesterol was measured with Amplex Red (Molecular Probes). Microscopy. Animals were pressure-perfused with 0.9% NaCl solution, brachiocephalic arteries were harvested, embedded and stained with hematoxylin/eosin or processed for immunohistochemical analysis according to standard protocol. The following antibodies were used: goat anti-mouse Sdc-1 (N-18, Santa Cruz), rat anti-mouse Sdc-1 (281-2, BD), rat anti-mouse macrophage (Mac3, BD), rabbit anti-mouse iNOS (ab15323, Abcam). iNOS positive plaque area was calculated using Image Pro Plus, data represent mean ± SEM, N=3 mice per genotype. Necrotic core was identified as the part of the lesion which appeared to be acellular (hematoxylin-negative); measurements were made using NIH ImageJ software, results were reported as %necrotic area/lesion area, N=10/genotype. To visualize the actin cytoskeleton in cultured cells, Sdc-1+/+ or Sdc-1−/− macrophages were fixed with 10% formalin and permeabilized with 0.5% Triton X-100. Cell were stained with Alexa Fluor 568 conjugated phalloidin (Invitrogen), nuclei were stained with DAPI. Slides were viewed using confocal microscopy.

Human tissue. Specimens consisting of human carotid plaque tissue were collected at Emory University Hospital under an approved IRB protocol. Tissue was fixed in 10% formalin and processed in paraffin, 5 µm sections were stained for macrophage (mouse anti-human CD68, PG-M1, Dako) and Sdc-1 (mouse anti-human DL101, Santa Cruz).

Cell culture and biochemical assays. Murine peritoneal macrophages were isolated from lavage at 4 or 5 days post thioglycollate injection. Cells were cultured according to standard protocol in DMEM. Where indicated, macrophage activation was induced as follows: M1 (100 U/ML IFN-γ, 100 ng/mL LPS); M2a (20 ng/mL IL-4); M2c (100 ng/mL IL-10); Sdc-1 (100 µM 6-Bnz-cAMP or 100 µM adenosine). Macrophage motility and phenotypic profile were identical for cAMP- or adenosine-induced Sdc-1 expression. Following activation, macrophages were harvested using non-enzymatic cell dissociation buffer (Sigma). Human acute monocytic leukemia THP1 cells were obtained from ATCC and cultured according to standard protocol in RPMI. Differentiation was induced by incubation with 50 ng/mL phorbol 12-myrisate 13-acetate (PMA) for 3 days. Activation was performed as described above. Griess reagent kit (Invitrogen) was used to assay iNOS activity. Briefly, 150 µL of 0.22 µm-filtered conditioned media was compared to a standard curve according to manufacturer instructions. Arginase activity was monitored in 4x10⁵ cells lysed with 0.1% Triton X-100, MnCl₂ was added to lysates at a final concentration of 1 mM. Arginase was activated by heating the mixture to 55°C for 10 min with 50 mM L-arginine. Mixtures were then incubated at 37°C for 60 min and reactions stopped with
the addition of H$_2$SO$_4$/H$_3$PO$_4$. Absorbance was measured at 540 nm after addition of α-isonitrosopropiophenone at 100°C for 30 min. Urea production was calculated against a standard curve and reported as fold change over un-stimulated control macrophages. IL-10 ELISA was performed according to manufacturer’s instructions (SA Biosciences), and following polarization stimulus, cells were washed and conditioned media was collected at 24 h.

Flow cytometry. Staining was performed according to standard protocol. The following antibodies were used for mouse studies: anti-macrophage (F4/80, BM8, eBioscience), anti-Sdc-1 (281-2, BD), anti-I-A/I-E (M5/114.15.2, BD), anti-PD-L2 (TY25, BD), anti-CD86 (GL1, BD). The following antibodies were used for human studies: anti-HLA-DR (G4606, BD), anti-Sdc-1 (MI15, BD).

PCR expression analysis. The inflammatory signature was characterized using the RT² profiler PCR array (SABiosciences mouse chemokines and receptors). RNA was TRizol extracted from classically activated M1 and MACS Sdc-1⁺ enriched (Miltenyi Biotech) macrophages and purified using RNeasy (Qiagen). A total of 1.0 µg of RNA was converted to cDNA using the RT² first strand kit (SABiosciences). qPCR was performed on the Applied Biosystems 7900 and the mRNA expression level for each gene was normalized to GAPDH according to a standard protocol. Reported values represent mean fold increase (M1 to Sdc-1⁺) from 3 independent experiments.

Phagocytosis index. Percent phagocytosis was determined by incubating macrophages with fluorescently labeled microspheres (1 µm diameter, Polysciences) at a ratio of 1:15 for 2 h at 37°C. Excess microspheres were removed by 3x wash in PBS and macrophages were harvested for flow cytometry using non-enzymatic cell dissociation buffer.

Efferocytosis. Apoptotic macrophages were generated by 5 min exposure of plated cells to ultraviolet light and a 90% apoptotic ratio was confirmed using flow cytometry with an annexin V/PI stain. Apoptotic macrophages were added to freshly isolated peritoneal macrophages (15:1) with a 2 h incubation, cells were washed and incubated in fresh culture media up to 40 h, and expression was monitored using flow cytometry, qPCR, and immunofluorescent staining (anti-syndecan-1 clone 281-2 PE, apoptotic cells were pre-labeled with cell trace CSFE green).

Migration and Adhesion. Leukocyte migration experiments were performed with peripheral blood mouse monocytes obtained by negative selection using a mouse monocyte enrichment kit (Stem Cell Technologies), according to manufacturer’s instruction and CD4⁺ T cells negatively selected from mouse splenocytes (Miltenyi Biotech). Prior to migration experiments, CD4⁺ cells were activated using dynabeads mouse T-activator CD3/CD28 (Invitrogen) and IL-12 (20 ng/mL). A total of 1x10⁵ monocytes or T cells in DMEM and 0.5% FBS were loaded into the upper chamber of 5 µM transmembrane supports (Corning) with macrophage conditioned media placed in the lower chamber. Conditioned media was generated from polarized macrophage populations cultured in DMEM and 0.5% FBS for 18 h. Macrophage migration experiments were performed using 1x10⁵ cells in 8-µm transwell plates (Corning) with DMEM containing 0.5% FBS. Cells were polarized on the transwell insert prior to the start of migration experiments to 10% FBS, apoptotic cell conditioned media, or apoptotic cells. Apoptotic conditioned media was collected after 4 h incubation with 5 min UV irradiated macrophages (90% apoptotic status confirmed with flow cytometry staining for annexin V/PI). At 18 h, cells were scraped from the upper surface of the membrane and macrophages that migrated to lower surface of the membrane were visualized with hematoxylin and counted (3 independent views/membrane). In migration experiments toward apoptotic cells, cells seeded to
the upper chamber were labeled with cell trace violet and apoptotic cells were labeled with cell trace CSFE according to manufacturer protocol; upon completion of migration, upper surface was scraped and total number of migrated cells (violet) were counted on the lower membrane and compared to total number of migrated cells with engulfed (violet + green) apoptotic cells. For each experiment, peritoneal macrophages were obtained from separate mice and 3 wells were tested. A migration index was calculated by dividing data obtained from experimental wells by those in control wells with basal media without stimulus. For adhesion experiments, macrophages were labeled with 1.5 µM Cell Trace CFSE (Invitrogen) and seeded to 96-well plates coated with fibronectin. Cells were cultured for 1 h, non-adherent cells were removed, adherent macrophages were lysed, and fluorescent signal measured and compared to a standard curve generated from known concentrations of CFSE labeled macrophages.

**Statistical analysis.** Data are expressed as mean ± SEM. Statistical differences were assessed using a 2-tailed t test or ANOVA. A P value less than 0.05 was considered statistically significant.
REFERENCES


Supplement Figure I. Flow cytometry scatter plot illustrating syndecan-1 staining in elicited peritoneal macrophages plated in basal media.
Supplement Figure II. Syndecan-1 protein expression is up-regulated following efferocytosis of apoptotic macrophages. CFSE labeled apoptotic macrophages (green) were added to plated adherent macrophages at a ratio of 10:1 for 2 h, cells were washed and stained with anti-syndecan-1 PE (C and D) or PE labeled isotype control (A and B) and Dapi nuclear counterstain (blue). Scale bar 100 µm.
Supplement Figure III. Control data is provided for boyden chamber migration of monocytes or CD4 T cells toward conditioned media generated from M1-polarized or Sdc-1-polarized macrophages, BM (basal DMEM media, 0.5% FBS), M1 (M1 conditioned media), Sdc1 (Sdc1 polarized conditioned media). Data are reported as % of cells that migrated into the bottom well, N=3.
Supplement Figure IV. Migration index of IL4 polarized (M2a) and IL10 polarized (M2c) sdc-1\(^{+/+}\) and sdc-1\(^{-/-}\) macrophages to serum.
Supplement Figure V. Control data is provided for boyden chamber migration toward apoptotic conditioned media, BM (basal DMEM media, 0.5% FBS), CM (apoptotic conditioned media). At 18 h, cells were scrapped from the upper surface of the membrane and macrophages that migrated to lower surface of the membrane were visualized with hematoxylin and counted (3 independent views/membrane).
Supplement Figure VI. Sdc-1\(^{-/}\) macrophages display normal M1/M2 polarization. A, Sdc-1\(^{-/}\) macrophages were treated with M1 (IFN\(\gamma\)/LPS), M2a (IL4), M2c (IL10), or adenosine and examined for surface expression of M1 (MHCII, CD86) or M2a (PD-L2) markers, data is displayed as histogram against the IgG control (red). As observed in sdc-1\(^{+/+}\) macrophages, sdc-1\(^{-/}\) M1 cells were MHCII\(^{\text{HIGH}}\) and CD86\(^{\text{HIGH}}\) and M2a cells were PD-L2\(^{\text{HIGH}}\). B, Elevated M1 iNOS activity was characterized via Griess reaction. C, IL-10 ELISA was measured in conditioned media. D, % phagocytosis was characterized in polarized populations by fluorescent bead uptake. Data are representative of triplicate sample mean ± SEM, \(*P<0.05.\)
Supplement Figure VII. Representative curve details resolution analysis at maximum macrophage infiltration ($\Psi_{\text{max}}$), at 50% macrophage resolution ($\Psi_{50}$) and at 25% macrophage resolution ($\Psi_{25}$).
Supplement Figure VIII. Immunohistochemical staining for sdc-1 in ApoE<sup>−/−</sup>Sdc-1<sup>−/−</sup> aortic tissue is provided as a control.
Supplement Figure IX. Representative brachiocephalic lesions from (A) ApoE^-/- and (B) ApoE^-/-Sdc-1^-/- mice after 8 weeks on Western diet. C, Lesion area measurements. Scale bars, 200 µm, data are mean ± SEM.
Supplement Figure X. Sdc-1 expression is consistent with M2 polarization and enhanced motility in human THP1 macrophages. A, M1 marker HLA-DR is expressed on IFNγ/LPS treated human macrophages but absent on cAMP treated macrophages. Sdc-1 expression is absent on M1 macrophages, like murine macrophages, sdc-1 expression is induced with cAMP. B, Sdc-1 polarized human macrophages display enhanced motility. Data are mean ±SEM, *P<0.05.