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S100A8 and S100A9 in Cardiovascular Biology and Disease

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Abstract

There is recent and widespread interest in the damage associated molecular pattern molecules S100A8 and S100A9 in cardiovascular science. These proteins have a number of interesting features and functions. For example, S100A8/A9 have both intracellular and extracellular actions, they are abundantly expressed in inflammatory and autoimmune states primarily by myeloid cells, but also by other vascular cells, and they modulate inflammatory processes, in part through toll-like receptor 4 (TLR4) and the receptor for advanced glycation end products (RAGE). S100A8/A9 also have anti-inflammatory and immune regulatory actions. Furthermore, increased plasma levels of S100A8/A9 predict cardiovascular events in humans, and deletion of these proteins protects *Apoe*$^{-/-}$ mice from atherosclerosis. Understanding the roles of S100A8 and S100A9 in vascular cell types, and the mechanisms whereby these proteins mediate their biological effects may offer new therapeutic strategies to prevent, treat, and predict cardiovascular diseases.

Introduction

S100A8 (calgranulin A or migration inhibitory factor-related protein 8; MRP-8) and its binding partner S100A9 (calgranulin B or MRP-14) are members of the S100 calcium-binding family of proteins, which are increased in a number of inflammatory and autoimmune states.[1] S100A8 and S100A9 form a heterocomplex, termed S100A8/A9 or calprotectin, but the two proteins may also have distinct functions and are regulated in part by different mechanisms.[2] The role of S100A8 and S100A9 in biology and disease is complex.[3–7] S100A8/A9 are generally viewed as inflammatory, but further studies have revealed both anti-inflammatory and immune regulatory actions.[2,6–7] The ability of S100A8/A9 to modulate inflammatory processes appears to be both context- and cell type-specific, suggesting an intricate network of regulation. Another layer of complexity surrounding the actions of S100A8/A9 is that these proteins have both intracellular and extracellular functions. The intracellular functions include calcium- and arachidonic acid binding, and regulation of microtubuli.[8–9] Released S100A8/A9 exert extracellular functions, some of which are mediated by toll-like receptor 4 (TLR4),[10] the receptor for advanced glycation end products (RAGE),[11] or other receptors.[12] S100A8/A9 are released from damaged/dying or activated cells through an atypical pathway that appears to require protein kinase C[13] and RAGE,[14] and S100A8 and S100A9 are therefore included in the group of proteins termed damage associated molecular pattern (DAMP) molecules.[6]

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Recently, S100A8/A9 were found to be of significance in cardiovascular disease both in humans and mice. In this review, we discuss S100A8/A9 as mediators of biological effects in the cardiovascular system with a special focus on atherosclerosis and cardiac dysfunction, and S100A8/A9 as markers of cardiovascular events.

What cardiovascular cell types express and release S100A8/A9?

Many cell types influence cardiovascular disease progression. Understanding which cells express and release S100A8/A9 is one of the first steps in elucidating the cardiovascular effects of S100A8/A9. Constitutive S100A8/A9 expression is believed to be limited to neutrophils and monocytes. This expression is tightly regulated throughout hematopoietic development and is lost during macrophage maturation. However, recent reports have highlighted the maintained expression of S100A8/A9 in some mature myeloid cell populations, and the induction of S100A8/A9 expression in non-myeloid cardiovascular cell types, as discussed below. These cell populations play distinct roles in cardiovascular biology and disease, and are affected by S100A8/A9 in different ways (Figure 1).

Expression of S100A8/A9 in hematopoietic development, neutrophils and monocytes

S100A8 and S100A9 are predominantly expressed in, and released from, myeloid cells upon cellular activation.[1,15–16] An early study of S100A9 gene expression in human cells demonstrated the tight regulation of S100A9 gene expression in a differentiation- and lineage-specific manner within the hematopoietic system.[17] Primitive, uncommitted $CD34^+CD38^-CD33^-$ and early myeloid $CD34^+CD33^+$ progenitor cells lack expression of S100A9. During monocyte maturation, up-regulation of S100A9 correlates with the expression of CD11b and CD14. Within the neutrophilic pathway, S100A9 is only detectable in $CD15^+$ cells, in which it slightly precedes and then correlates with CD11b expression. Mature $CD15^+CD16^+CD33^+$ neutrophils express S100A9, indicating that S100A9 expression is initiated during maturation of promyelocytes towards myelocytes and then maintained up to the level of mature neutrophils. Similar patterns of expression are observed in murine myeloid development.[18–19] Other lineage hematopoietic cells, including the $CD19^+$ B-lineage, $CD3^+$ T-lineage and GlyA-positive erythroid cells, are S100A9 negative,[17] although recent reports describe S100A8 and S100A9 gene expression in a highly purified B-cell population[20] and in platelets.[21]

S100A8 and S100A9 constitute approximately 40% of the cytosolic protein fraction in circulating human blood neutrophils and 1% in monocytes.[16] Although expression is downregulated during maturation of peripheral blood monocytes to tissue macrophages, expression can be found in macrophages in inflamed tissues.[22–24]

Expression of S100A8/A9 in other myeloid-derived cell populations

S100A8/A9 are expressed in dendritic cells (DCs) and myeloid-derived suppressor cells (MDSCs). Although S100A8/A9 expression diminishes during differentiation into DCs[25] some expression is maintained in DCs.[26] Moreover, gene expression of S100A8 and S100A9 is significantly upregulated in mature human DCs after IL-10 treatment.[27] In contrast to macrophages, DCs constitutively release S100A8/A9.[26] MDSCs also synthesize and secrete S100A8/A9, which in turn activate RAGE and promote MDSC migration.[28] These cells accumulate in tumor-bearing hosts and in response to inflammation, where they inhibit T and NK cell activation and DC differentiation. Another myeloid-derived cell population termed fibrocytes express S100A8/A9.[29] Human fibrocytes are believed to be derived from circulating $CD14^+$ monocytes, express typical macrophage markers, and to play a role in tissue repair and fibrosis.[29] The roles of MDSCs and fibrocytes in cardiovascular disease are unknown.

Expression of S100A8/A9 in non-myeloid cells

The hallmark of S100A8 and S100A9 expression in non-myeloid cells is their gene induction in response to stress. There is little S100A8/A9 in endothelial cells and vascular smooth muscle cells (VSMCs) under normal conditions. Expression in endothelial cells can be induced after activation with LPS, IL-1β, or TNF-α,[26,30] or after exposure to elevated glucose levels *in vitro* or diabetes *in vivo*.[31] No detectable release of S100A8/A9 has been found from cultured endothelial cells.[26,30] In VSMCs, S100A9 expression is induced by the gram-negative bacterium *Porphyromonas gingivalis*,[32] and S100A8/A9 expression is stimulated by LPS in cardiomyocytes.[11] Together, these studies show that S100A8/A9 expression can be induced in non-myeloid cardiovascular cells through inflammatory stimuli likely to be present in atherosclerotic lesions and other cardiovascular pathologies.

S100A8/A9 release from intact non-myeloid cells is significantly lower than that from myeloid cells, suggesting that the biological effects of S100A8/A9 expressed by these cells might be largely intracellular, unless membrane integrity is compromised. Accordingly, Croce *et al.* demonstrated that the effects of S100A8/A9 on VSMC proliferation are mediated by intracellular actions.[33]

S100A8/A9 play important roles in atherosclerosis and vascular injury

Human studies

Monocytes play important roles in all stages of atherosclerosis in humans and mice.[34] It was recently shown that the human $CD14^+CD16^-$ monocyte population expresses more S100A8 than does the $CD14^+CD16^+$ monocyte population, similar to the elevated expression of S100A8 in mouse Ly-$6C^+$ monocytes, as compared to Ly-$6C^{lo}$ monocytes.[35] In mice, the Ly-$6C^+$ monocyte population preferentially infiltrates lesions of atherosclerosis,[36–37] but the role of different monocyte populations in human atherosclerosis is not well understood. Within human lesions, S100A9 immunoreactivity is associated with macrophages, microvessels and calcified areas.[38] A subsequent study demonstrated that the percentage of S100A8/A9-positive macrophages is higher in rupture-prone lesions, as compared to stable ones.[39] Furthermore, increased serum levels and expression of S100A8/A9 were observed in infiltrated neutrophils in atherosclerotic plaques of patients with unstable angina.[40]

The interest in S100A8/A9 in relation to cardiovascular disease increased markedly when Healy *et al.*[21] demonstrated that plasma levels of S100A9 among apparently healthy women predict the risk of future nonfatal myocardial infarction, nonfatal stroke, and cardiovascular death. S100A8/A9 was subsequently found to be an early marker for detection of acute coronary syndromes,[41] and the risk of a recurrent cardiovascular event was increased with each increasing quartile of S100A8/A9 in the PROVE IT-TIMI 22 trial.[42] Together, these studies demonstrate that plasma levels of S100A8/A8 appear to be a marker of cardiovascular risk in humans.

Mouse studies

Monocyte populations that infiltrate atherosclerotic lesions in *Apoe*$^{-/-}$ mice express S100A9.[43] Studies in mice have also demonstrated that S100A8/A9 are upregulated in macrophages overexpressing urokinase plasminogen activator concomitant with increased plaque rupture in LDL receptor (LDLR)-deficient (*Ldlr*$^{-/-}$) mice,[44] and that S100A8 gene expression is reduced in macrophages from regressing lesions.[45] Together, these studies suggest that S100A8/A9 are upregulated in activated macrophages *in vivo*. Interestingly, a connection to diabetic vascular disease was established with the findings that macrophages from diabetic *Ldlr*$^{-/-}$ mice express and secrete higher levels of S100A9 as compared to macrophages from non-diabetic mice,[46] and that elevated glucose levels result in increased

expression of S100A8 in isolated macrophages.[45] These results are corroborated by findings of increased S100A9 immunoreactivity in macrophage-rich lesions in these diabetic mice[46] and in diabetic *Apoe*$^{-/-}$ mice.[47]

Mouse models have been used to investigate whether S100A8/A9 play causative roles in atherosclerosis. An interesting study on *S100a9*$^{-/-}$*;Apoe*$^{-/-}$ mice demonstrates that this is indeed the case.[33] These double knockout mice had an approximate 30% reduction in *en face* aortic lesion area in response to a high-fat diet, as compared to *Apoe*$^{-/-}$ controls. This reduction was not due to differences in plasma lipids, but to a reduced accumulation of lesion macrophages.[33] The same study showed that both neointimal thickening following femoral artery injury, and lesions in a model of thrombohemorrhagic vasculitis are reduced in *S100a9*$^{-/-}$ mice.[33] Interestingly, accumulation of both monocytes and neutrophils was reduced in *S100a9*$^{-/-}$ mice, suggesting that S100A8/A9 promote accumulation of both cell types at sites of vascular injury and atherosclerosis.

Based on these findings and the concept that a majority of S100A8/A9 is derived from myeloid cells, we hypothesized that the protective effects of S100A9-deficiency on atherosclerosis would be mimicked by S100A9-deficiency specifically in bone marrow-derived cells. We therefore undertook a study in which *Ldlr*$^{-/-}$ mice were transplanted with bone marrow from *S100a9*$^{-/-}$ mice or wildtype littermate controls and then fed a high-fat diet for 20 weeks.[26] Surprisingly, neither atherosclerosis nor macrophage accumulation in lesions was affected by bone marrow S100A9-deficiency. Lesion neutrophils were not abundant in this study.[26] The lack of effect of bone marrow S100A9-deficiency on atherosclerosis might be due to the production and secretion of S100A8/A9 at sufficiently high levels from non-bone marrow-derived cells, that other DAMPs or their receptors compensate for the loss of bone marrow-derived S100A8/A9, or that intracellular S100A8/A9 levels in non-myeloid cell types play a more important role than previously recognized. The latter possibility is supported by data describing S100A8/A9 expression in both VSMCs and endothelial cells, and “pro-atherosclerotic” effects of S100A8/A9 in these cell types.[32,33,48] However, we cannot exclude the possibility that S100A8/A9 might be relatively more important mediators in atherosclerosis in *Apoe*$^{-/-}$ mice as compared to *Ldlr*$^{-/-}$ mice.

A more significant question is whether S100A8/A9 promote atherosclerosis and cardiovascular disease in humans. New tools, such as S100A8/A9 neutralizing antibodies or specific inhibitors of S100A8/A9 secretion, will have to be developed and proved safe before this important question can be addressed. S100A8 and S100A9 polymorphism studies[49] might also shed additional light onto the roles of these proteins in cardiovascular disease in humans.

S100A8/A9 and cardiac dysfunction

S100A8/9 have important functions in the injured heart, especially in contributing to cardiovascular dysfunction as a result of sepsis. *S100a9*$^{-/-}$ mice are largely protected from endotoxin-induced cardiomyocyte dysfunction, measured as reduced ejection fraction.[11] The effects of S100A8/A9 appear to be mediated by altered calcium flux following RAGE activation, since both cardiac S100A8 and S100A9 were found to co-immunoprecipitate with RAGE following LPS injection, and RAGE blockade abolished the decreased calcium flux by S100A8 or S100A9.[11] On the other hand, in a rat model of experimental autoimmune myocarditis, treatment with recombinant human S100A8/A9 results in improved left ventricular ejection fraction, reduced infiltration of immune cells, and reduced levels of cytokines, as compared to saline-injected controls.[50] Thus, the role of S100A8/A9

in the heart might depend on the stimuli and the contribution of RAGE or other receptors. The role of S100A8/A9 in the human heart is unknown.

Is S100A8/A9 pro- or anti-inflammatory in cells contributing to cardiovascular disease?

As discussed above, S100A8/A9 can activate TLR4 and RAGE, indicating a pro-inflammatory role for extracellular S100A8/A9. However, anti-inflammatory effects of these S100A proteins have also been described, which might be mediated by oxidation and/or S-nitrosylation of the S100A proteins,[7] arachidonic acid binding,[8] or other effects. Furthermore, it is becoming increasingly clear that S100A8/A9 have different functions in different cell types involved in atherosclerosis (Figure 1). For example, S100A8/A9 promote an inflammatory phenotype in neutrophils, while suppressing an inflammatory phenotype in DCs.[26] We thus proposed that the overall function of S100A8/A9 on atherosclerosis depends on the relative levels of cell types involved in the disease process.[26] What evidence supports cell type-specific effects of S100A8/A9 in vascular cells?

Neutrophils

Neutrophils are present in lesions of atherosclerosis, although at relatively levels lower than those of monocytes/macrophages.[26,51–52] S100A8/A9 are abundant in neutrophils, and not surprisingly, many of the effects of S100A8/A9 have been described in neutrophils, in which S100A8/A9 are pro-inflammatory, at least in part through extracellular activation of TLR4 (Figure 2).[10,26] The ability of S100A9 to promote phagocytosis in neutrophils has also been attributed to extracellular activation of TLR4, RAGE, or another receptor.[53] Accordingly, neutrophils from *S100a9*$^{-/-}$ mice show decreased activation of NADPH oxidase, an enzyme involved in pathogen killing following phagocytosis, as compared to wild-type mice.[54] This effect, however, appears to be mediated by intracellular S100A8/A9 activating NADPH oxidase through their intracellular arachidonic acid-binding activity (Figure 2).[54] Furthermore, intracellular S100A8/A9 contribute to neutrophil CD11b surface expression, neutrophil adhesion, and migration into inflamed tissues through calcium and microtubule regulation.[9,55] Thus, neutrophil S100A8/A9 mediate phagocytosis and inflammatory effects by both extracellular and intracellular pathways.

Monocytes/macrophages

S100A8/A9 levels in monocytes are ~40-fold lower than those of neutrophils.[16] Extracellular S100A8/A9 enhance the inflammatory cytokine production by human monocytes,[56] and monocytes from *S100a9*$^{-/-}$ mice exhibit a reduced ability to migrate toward chemokines.[33] Furthermore, mouse *S100a9*$^{-/-}$ peritoneal macrophages isolated 48 h after thioglycollate-injection have an impaired ability to release cytokines following LPS-stimulation.[33] However, in mature peritoneal macrophages isolated 5 days after thioglycollate-injection, S100A9-deficiency does not affect cytokine release in response to LPS.[26] It is well-known that as monocytes differentiate into macrophages, S100A8/A9 expression is markedly downregulated.[23–24,57] Therefore, it is possible that the distinct responses to loss of endogenous S100A8/A9 between monocyte/early macrophages and mature macrophages are due to differences in S100A8/A8 expression. Another interesting possibility, based on forced S100A9 overexpression studies, is that S100A9 might delay myeloid differentiation,[58] possibly explaining the different effects of S100A8/A9-deficiency in monocytes and different states of macrophage differentiation. The mechanisms whereby S100A8/A9 promote an inflammatory phenotype of monocytes/macrophages involve activation of the NF-κB and p38 MAPK pathways.[56] It is tempting to speculate that this is due, at least in part, to activation of TLR4 and/or RAGE. S100A8/A9-mediated stimulation of monocyte adhesion has also been attributed to extracellular effects.[59] On the other hand,

S100A9 released in macrophages from phagocytosed apoptotic neutrophils has been proposed to inhibit macrophage activation through S100A9's calcium-binding activity.[60] These findings suggest that, like in neutrophils, S100A8/A9 have both extracellular and intracellular effects in monocytes/macrophages (Figure 2), and that the significance of endogenous S100A8/A9 depends on the expression levels in different monocyte/macrophage maturation and activation states.

Dendritic cells

DCs contribute significantly to atherosclerosis, at least in mice.[61–62] DC differentiation is blocked by S100A9 overexpression, and it has been shown that S100A8/A9-overexpressing DCs have a lower ability to stimulate the proliferation of allogeneic T cells than do control DCs.[58] The same study[58] showed that overexpression of S100A9 in hematopoietic cells in mice results in accumulation of myeloid progenitors at the expense of DC and macrophage differentiation, and suggested that the effects of S100A9 were due to increased reactive oxygen species. Consistent with these results, *S100a9*$^{-/-}$ DCs promote a higher T cell proliferation compared to wild-type DC in mixed lymphocyte reactions.[26] In addition, *S100a9*$^{-/-}$ DCs exhibit increased release of cytokines following stimulation with TLR2 or TLR4 ligands, as compared to DCs from wildtype littermates,[26] and express more DC cell surface markers, (CD205, IA) and co-stimulatory molecules (CD40, CD86) compared to wild-type cells (unpublished data, 2011). Because TLR4 and RAGE activation is thought to promote DC maturation,[63–64] S100A8/A9 might act through mechanisms distinct from TLR4 and RAGE to suppress inflammatory effects in DCs, perhaps indirectly by suppressing DC differentiation.

Endothelial cells

Exogenous S100A8/A9 increase expression of adhesion molecules, such as VCAM-1 and ICAM-1, and chemokines, as well as permeability in endothelial cells.[48] Together, these effects could promote infiltration of immune cells into inflamed tissues.

S100A8/A9 are part of a larger network of proteins with cardiovascular effects

S100A8 and S100A9 belong to a multigenic family of S100 proteins that are differentially expressed in a wide variety of cell types.[65] The functional diversity of S100 proteins is achieved by their specific cell- and tissue-expression patterns, structural variations, different metal ion binding properties, as well as their ability to form homo-, hetero- and oligomeric assemblies.[66] Some S100 proteins, in particular S100A8, S100A9, and S100A12, are released from cells as a result of cellular activation. In the extracellular milieu, they function as ligands of pattern-recognition receptors, such as TLR4[10] and RAGE,[11] and possibly other receptors, as discussed above. Both TLR4[67] and RAGE[68–69] have been found to promote atherosclerosis in mice, although the effect of TLR4-deficiency is not consistently observed.[70–71]

Beside S100A8 and S100A9, S100A12, S100B, and S100A4 are implicated in the pathogenesis of atherosclerosis. S100A12 has been described as an endogenous RAGE ligand with pro-inflammatory functions in humans[72–73], but is not expressed in the mouse.[74] Elevated S100A12 levels are found in ruptured coronary artery plaques in patients suffering sudden cardiac death[75] and in sera from patients with coronary artery disease.[76] The latter study did not detect increased cytokine production in human monocytes or macrophages stimulated with human S100A12,[76] suggesting that S100A12 actions on inflammation might be as complex as those of S100A8/A9. However, a recent study demonstrated that expression of human S100A12 in VSMCs results in increased atherosclerosis and more

calcification of lesions in *Apoe*$^{-/-}$ mice.[77] The S100A12 transgene also elicited the expression of genes involved in osteogenesis by cellular pathways that were dependent on RAGE and oxidative stress signaling. These findings suggest a causative involvement of S100A12 in atherosclerosis and vascular calcification.[77]

Another S100 protein, S100A4, exerts growth-promoting effects in VSMCs[78] and, therefore, might play a role in atherogenesis. In humans, S100A4 is barely detectable in coronary artery media, but is markedly expressed in VSMCs of atheromatous and restenotic coronary artery lesions.[78] There is no direct evidence available for S100A4/RAGE interaction.

Besides its abundance in astrocytes, S100B is expressed in cells outside the brain, including in DCs and VSMCs.[79] S100B exerts effects on both endothelial cells and VSMCs that might have an impact on atherosclerosis. These effects are mainly RAGE-dependent.[80] In human endothelial cells, S100B up-regulates the gene expression of monocyte chemoattractant protein 1 (MCP-1), and RAGE.[81] In VSMCs, S100B has been found to stimulate IL-6 and MCP-1 production and cell migration.[82]

Very recently, elevated serum levels of S100B, S100A6 and S100P were found to be associated with the acute coronary syndrome, and serum levels and myocardial expression of these proteins were related to infarct size.[83] Thus, the group of S100 protein family members with potential roles in cardiovascular biology and disease is rapidly expanding.

Importantly, these S100 proteins, other DAMPs, and their receptors are likely to impact each other and to synergize *in vivo*. As an example, activation of RAGE results in increased expression and release of S100A8 and S100A9,[14] and AGEs exacerbate the pro-inflammatory effects of S100A8/A9,[84] contributing to a likely positive feedback loop in states of inflammation. Similarly, in the setting of diabetes, DAMPs, RAGE, and TLR4 are all upregulated in monocytes/macrophages,[47,85] potentially contributing to a "perfect storm" of activation of this network of proteins.

Many questions remain unanswered

We have reviewed the evidence that S100A8/A9 are both biomarkers and mediators of cardiovascular disease. However, more research is needed to elucidate the complex effects of S100A8 and S100A9. For example, to what extent are the biological effects of S100A8/A9 mediated by intracellular versus extracellular actions?; why and how do S100A8/A9 mediate pro-inflammatory and anti-inflammatory effects in different cell types and disease states?; what is the relative contribution of S100A8/A9 versus other DAMPs?; which are the cell types that contribute to S100A8/A9 expression and secretion in different disease states; and to what extent do S100A8 and S100A9 contribute to cardiovascular disease in humans? Answers to the questions above are needed before S100A8/A9 can be considered as therapeutic targets.

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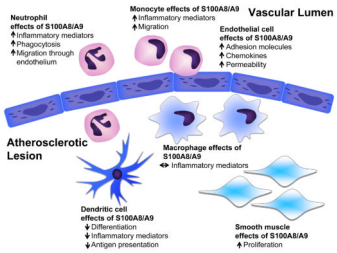

Figure 1. Schematic representation of the potential effects of S100A8/A9 in atherosclerosis

In neutrophils, S100A8/A9 promote expression of inflammatory mediators, phagocytosis, and migration through the vascular endothelium. Likewise, in monocytes, S100A8/A9 stimulate migration and an inflammatory phenotype. Endothelial cells express S100A8/A9 following exposure to inflammatory stimuli, and when added exogenously, these proteins promote adhesion molecule expression, chemokine expression, and permeability of the endothelial layer, all of which may promote atherogenesis. Macrophages express and release significantly less S100A8/A9 than do DCs. S100A8/A9 suppress DC differentiation, antigen presentation, and release of inflammatory mediators. S100A8/A9 promote proliferation of VSMCs, which might contribute to fibrous cap formation. Thus, S100A8/A9 affect the major cell types involved in atherosclerosis, and these proteins have cell type-selective effects. The relative contribution of these cell types during different stages of lesion progression is likely to govern the overall effect of S100A8/A9 inhibition.

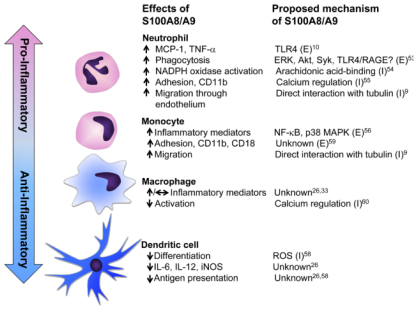

Figure 2. S100A8/9 differentially modify phenotypic states of neutrophils, monocytes/ macrophages, and dendritic cells through extracellular and intracellular mechanisms

The effects of S100A8/A9 on processes involved in inflammation, and the proposed signaling pathways are shown for different myeloid-derived cells. (E), extracellular effects; (I), intracellular effects of S100A8/A9