S100A9 Differentially Modifies Phenotypic States of Neutrophils, Macrophages, and Dendritic Cells
Implications for Atherosclerosis and Adipose Tissue Inflammation

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Background—S100A9 is constitutively expressed in neutrophils, dendritic cells, and monocytes; is associated with acute and chronic inflammatory conditions; and is implicated in obesity and cardiovascular disease in humans. Most of the constitutively secreted S100A9 is derived from myeloid cells. A recent report demonstrated that mice deficient in S100A9 exhibit reduced atherosclerosis compared with controls and suggested that this effect was due in large part to loss of S100A9 in bone marrow–derived cells.

Methods and Results—To directly investigate the role of bone marrow–derived S100A9 in atherosclerosis and insulin resistance in mice, low-density lipoprotein receptor–deficient, S100A9-deficient bone marrow chimeras were generated. Neither atherosclerosis nor insulin resistance was reduced in S100A9-deficient chimeras fed a diet rich in fat and carbohydrates. To investigate the reason for this lack of effect, myeloid cells were isolated from the peritoneal cavity or bone marrow. S100A9-deficient neutrophils exhibited a reduced secretion of cytokines in response to toll-like receptor–4 stimulation. In striking contrast, S100A9-deficient dendritic cells showed an exacerbated release of cytokines after toll-like receptor stimulation. Macrophages rapidly lost S100A9 expression during maturation; hence, S100A9 deficiency did not affect the inflammatory status of macrophages.

Conclusions—S100A9 differentially modifies phenotypic states of neutrophils, macrophages, and dendritic cells. The effect of S100A9 deficiency on atherosclerosis and other inflammatory diseases is therefore predicted to depend on the relative contribution of these cell types at different stages of disease progression. Furthermore, S100A9 expression in nonmyeloid cells is likely to contribute to atherosclerosis.

Key Words: atherosclerosis • immunology • macrophage • S100 proteins

S100A9 and its binding partner, S100A8, are members of the S100 family of proteins and are promising novel markers of cardiovascular risk in humans. Recent studies on mice demonstrate that S100A8/A9 promote atherosclerosis. Thus, S100A9 appears to be both a marker and a mediator of atherosclerosis. Furthermore, circulating levels of S100A8/A9 are increased in a number of autoimmune and proinflamatory states, including type 1 diabetes mellitus and obesity, characterized by increased cardiovascular risk. Studies on S100A9-deficient mice suggest that S100A8/A9 have proinflammatory actions, eg, in sepsis and pancreatitis.

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S100A8/A9 are abundantly and constitutively expressed in neutrophils and monocytes. Expression of S100A8/A9 is lost during differentiation of monocytes into macrophages, yet some expression is sustained in dendritic cells (DCs). S100A8/A9 promote migration of neutrophils through increased CD11b expression. The effects of S100A9 deficiency in neutrophils might be due to the combined loss of S100A8 and S100A9 because S100A9-deficient neutrophils express S100A8 mRNA but not S100A8 protein, suggesting that S100A9 stabilizes S100A8. S100A8/A9 are secreted from phagocytes through a tubulin-dependent mechanism, and extracellular functions include signaling through toll-like receptor (TLR)-4 and the receptor for advanced glycation end products resulting in proinflammatory effects. However, S100A8/A9 expression is also induced by antiinflammatory signals, and overexpression of S100A9 blocks maturation of DCs. Thus, S100A8/A9 might exert inhibitory or antiinflammatory actions.
Because of the abundance of S100A8/A9 in myeloid cells compared with nonmyeloid cells, myeloid-derived S100A8/A9 are believed to mediate the effects of whole-body S100A9 deficiency. However, S100A8 and S100A9 can be induced by inflammatory stimuli in nonhematopoietic cells, eg, endothelial cells. Therefore, the goal of this study was to investigate whether myeloid-derived S100A8/A9 contribute to atherosclerosis. Insulin resistance was analyzed as a secondary end point.

Our results demonstrate that bone marrow–derived S100A9 is not sufficient to promote atherosclerosis or insulin resistance in low-density lipoprotein receptor–deficient (LDLR−/−) mice. Furthermore, we have uncovered disparate effects of S100A9 on the inflammatory phenotype of neutrophils, macrophages, and DCs. These findings suggest that the biological effects of S100A9 depend on the relative abundance of different immune cells and nonmyeloid cells in different disease states.

Methods

S100A9-Deficient Mice

SV129:C57BL/6J S100A9−/− mice were backcrossed 10 generations onto the C57BL/6J background, and a colony of S100A9−/− mice was used to generate S100A9 wild-type (S100A9+/+) and S100A9−/− offspring. S100A9−/− mice were viable with normal myelopoietic properties, consistent with previous studies. Male and female mice (10 to 15 weeks of age) were used for cell isolation. In vivo experiments included studies on male whole-body S100A9−/− mice and bone marrow transplantsations from S100A9−/− and littermate S100A9+/+ donors into male LDLR−/− recipients. All animal studies were approved by the Institutional Animal Care and Use Committee at the University of Washington.

Cell Isolation

Peritoneal neutrophils and macrophages were collected 4 hours and 5 days after thioglycollate injection, respectively. Bone marrow–derived macrophages were differentiated in 30% L-conditioned medium (a source of macrophage colony-stimulating factor), and DCs were differentiated in 3 to 10 ng/mL recombinant granulocyte macrophage colony-stimulating factor for 5 to 7 days. In some experiments, monocyte enrichment from bone marrow was achieved by use of a negative-selection monocyte-enrichment kit (Easy Sep, Vancouver, BC, Canada). Classic activation of DCs and macrophages was induced by a 24-hour exposure to 5 ng/mL lipopolysaccharide (LPS) plus 12 ng/mL interferon-γ (IFNγ). To examine TLR activation, CD11+ DCs were exposed to various concentrations of zymosan, LPS, CpG-B, and Pam3CSK4 overnight. T cells from spleen and peripheral lymph nodes were collected with subsequent immunomagnetic depletion of contaminating cells, as described in the online-only Data Supplement. More than 95% of these cells were CD4+ or CD8+, as evaluated by flow cytometry.

Atherosclerosis and Insulin Resistance Studies

Male C57BL/6 whole-body S100A9−/− and S100A9−/− mice (10 to 12 weeks old) were fed a chow diet or a diabetogenic diet rich in fat and carbohydrates with 0.15% wt/wt cholesterol (DCC; BioServ No. F04997) for 12 weeks. Insulin resistance was evaluated by glucose and insulin tolerance tests at 10 and 12 weeks, respectively. The mice were then saline perfused, and epididymal fat was dissected, weighed, and fixed in parafomaldehyde.

After lethal irradiation, male LDLR−/− mice (12 to 14 weeks old) received intravenous bone marrow transplants from S100A9−/− or S100A9−/− mice. After a 3-week recovery period, the mice were fed chow or DCC for 24 weeks. Body weights were measured weekly, and blood glucose and blood cholesterol were determined at the beginning and end of the study. There were no differences in these measurements between the groups at baseline. Glucose and insulin tolerance tests were performed at 20 and 22 weeks, respectively. At the end of the 24-week study, mice were saline perfused under physiological pressure. Epididymal fat pads, heart, and the brachiocephalic artery were dissected, fixed, embedded, and serial sectioned. Every fourth section was stained with a Movat pentachrome stain. Adjacent sections were stained with antibodies against S100A9, Ly6G, Mac-2, and major histocompatibility complex class II. Maximal atherosclerotic lesion area was determined in a masked fashion. Plasma interleukin (IL)-6 was analyzed with ELISA, and triglycerides were determined with a kit from Sigma Aldrich (St Louis, MO).

Additional Methods

General laboratory methods and a more detailed description of methods are provided in the online-only Data Supplement.

Statistical Analyses

Prism4 (GraphPad Software, Inc, La Jolla, CA) was used for statistical analysis. Unpaired Student t test was used to compare 2 groups. One- or 2-way ANOVA was used to compare >2 groups or parameters with the Tukey and Bonferroni posthoc tests, respectively. Values of P<0.05 were considered statistically significant. All P values are based on 2-tailed analyses.

Results

Bone Marrow S100A9 Deficiency Does Not Diminish Atherosclerosis in LDLR−/− Mice

Whole-body S100A9 deficiency reduces atherosclerosis in apolipoprotein E–deficient (Apoe−/−) mice through reduced arterial macrophage accumulation. We therefore asked whether bone marrow–specific deletion of S100A9 would recapitulate the effect of whole-body S100A9 deficiency on atherosclerosis. Bone marrow from S100A9−/− and S100A9−/− mice was transplanted into irradiated male LDLR−/− mice. The mice were fed chow or DCC for 24 weeks to allow the development of atherosclerosis and insulin resistance (Figure 1A). Successful chimerism was confirmed by several different methods. Thus, S100A9 mRNA was undetectable in circulating blood cells from mice that had received S100A9-deficient bone marrow, whereas S100A8 mRNA levels were unchanged (Figure 1B). Accordingly, blood clots from LDLR−/− S100A9−/− bone marrow chimeras exhibited no detectable S100A9 immunoreactivity despite the presence of neutrophils identified by the neutrophil marker Ly6G, whereas clear S100A9-positive cells were identified in the wild-type controls (Figure 1C). Bone marrow cells from S100A9-deficient mice differentiated in the presence of granulocyte macrophage or macrophage colony-stimulating factor also showed loss of S100A9 mRNA and loss of both S100A9 and S100A8 protein (Figure 1D and 1E and Figure I in the online-only Data Supplement), consistent with published results.

DCC-fed LDLR−/− mice exhibited significant increases in plasma cholesterol and triglycerides compared with chow-fed controls, and there was no effect of bone marrow S100A9 deficiency on these parameters (Figure 2A and 2B). Atherosclerosis was analyzed in Movat pentachrome–stained cross sections from the aortic sinus and the brachiocephalic artery. DCC-fed mice demonstrated larger...
aortic sinus and brachiocephalic artery lesions than chow-fed mice, confirming previous reports (Figure 2C and Figure IIA in the online-only Data Supplement). However, bone marrow S100A9 deficiency did not modulate lesion area (Figure 2C and Figure IIA in the online-only Data Supplement). Immunohistochemistry was used to evaluate macrophage, DC, neutrophil, and T-cell accumulation in atherosclerotic tissue. The Mac-2–positive area (identifying both macrophages and DCs; L.B., M.M.A., K.E.B., and J.W.H., unpublished observations) in the aortic sinus and brachiocephalic artery was significantly elevated in the DDC-fed mice compared with chow-fed mice, with no effect of bone marrow S100A9 deficiency (Figure 2D and Figure IIB in the online-only Data Supplement). In brachiocephalic artery lesions, neutrophils were detected in only 1 of 18 DDC-fed mice (Figure III in the online-only Data Supplement), suggesting that neutrophils do not play a significant role in this model, at least not at this time point. Likewise, no CD3⁺ T cells were detected in sinus lesions (data not shown). Importantly, S100A9⁻/⁻ bone marrow chimeras had undetectable S100A9 immunoreactivity in Mac-2–positive areas in sinus lesions (Figure 2E and 2F), confirming successful chimerism. Finally, there were no differences in necrotic core size or other lesion morphological features between mice with S100A9⁺/⁺ and S100A9⁻/⁻ bone marrow (data not shown). Thus, S100A9 deficiency in bone marrow–derived cells is not sufficient to reduce atherosclerosis or accumulation of macrophages/DCs in LDLR⁻/⁻ mice.

**S100A9 Deficiency Does Not Affect Insulin Resistance**

Adipose tissue inflammation, with accumulation of immune cells, is believed to play a causal role in insulin resistance, and TLR4 expression on hematopoietic cells promotes this effect. Male DDC-fed LDLR⁻/⁻ mice develop insulin resistance, obesity, systemic inflammation, and macrophage infiltration into epididymal adipose tissue. We therefore investigated whether S100A9 deficiency would affect these parameters.

Chow-fed LDLR⁻/⁻ mice showed stable body weight, whereas DDC-fed mice gained a significant amount of weight during the 24-week study (Figure 3A) and exhibited increased epididymal fat pad weight (Figure 3B).
There was, however, no difference in weight gain or fat pad mass between LDLR<sup>−/−</sup> mice with S100A9<sup>+/+</sup> and S100A9<sup>−/−</sup> bone marrow (Figure 3A and 3B). Systemic inflammation, as detected by circulating IL-6, was increased in DDC-fed mice, but bone marrow S100A9 deficiency had no significant effect (Figure 3C). The epididymal Mac-2–positive area was significantly increased in DDC-fed mice compared with chow-fed mice (Figure 3D and 3E). However, there was no significant difference in mice with S100A9<sup>+/+</sup> and S100A9<sup>−/−</sup> bone marrow (Figure 3D).

To evaluate insulin resistance, we conducted glucose and insulin tolerance tests. DDC-fed mice had significantly elevated baseline glucose and increased insulin resistance compared with chow-fed mice (Figure 3F and 3G). Similar to the lack of difference in adipose inflammation and body weight, there was no effect of bone marrow S100A9 deficiency on glucose and insulin tolerance tests (Figure 3F and 3G).

Although S100A8 and S100A9 are expressed primarily in myeloid cells, evidence suggests that inflammatory stimuli induce their expression in nonmyeloid cells, including endothelial cells. Accordingly, in isolated mouse heart endothelial cells, S100A8 mRNA expression was increased after tumor necrosis factor-α treatment compared with controls (Figure IV in the online-only Data Supplement). This suggests that under proinflammatory conditions such as those present in diet-induced obesity, nonhematopoietic cells may express increased levels of S100A8/A9. Secreted S100A8/A9 from unstimulated or tumor necrosis factor-α–stimulated endothelial cells were below detection (data not shown), indicating that endothelial cells secrete lower amounts of these proteins than do myeloid cells or do not release these proteins unless damaged.

To assess the potential role of S100A8/A9 in nonhematopoietic cells, whole-body S100A9<sup>+/+</sup> and S100A9<sup>−/−</sup> C57BL/6J male mice were fed chow or DDC for 12 weeks. DDC-fed animals gained a significant amount of weight compared with chow-fed mice with no effect of S100A9 deficiency on body weight or fat pad weight (Figure 4A and 4B). After 11 to 12 weeks on DDC, the mice displayed glucose intolerance, yet whole-body S100A9 deficiency had no significant effect on insulin or glucose tolerance (Figure 4C and 4D). Although DDC-fed mice exhibited increased accumulation of Mac-2–positive cells in epidid-
Neutrophils, Early Differentiation Stages of Macrophages and DCs, and More Mature DCs Express Significantly More S100A9 Than Do Macrophages

To investigate the reason(s) for the unexpected lack of effect of bone marrow S100A9 deficiency on atherosclerosis, we performed a number of studies. First, expression levels of S100A9 in different myeloid cell populations were determined. S100A8 and S100A9 mRNA levels were analyzed in neutrophils, immature macrophages and DCs, and more mature macrophages and DCs in wild-type mice. S100A8 and S100A9 mRNA levels were markedly higher in thioglycollate-elicted neutrophils compared with thioglycollate-elicted macrophages (Figure 5A and 5B), consistent with previous studies. S100A9 protein levels were also higher in neutrophils compared with macrophages (Figure 5C). S100A8 and S100A9 mRNA levels declined rapidly during macrophage and DC differentiation (Figure 5D and data not shown), as expected.

Next, we investigated the levels of S100A8A9 in bone marrow–derived DCs differentiated in the presence of granulocyte macrophage colony-stimulating factor– compared with macrophage colony-stimulating factor–differentiated macrophages. The DC population expressed higher levels of the DC markers CD11c and major histocompatibility complex class II (Figure V in the online-only Data Supplement). DCs expressed more S100A8 and S100A9 mRNA and protein (Figure 6A through 6C) compared with macrophages. In parallel experiments, the relative levels of secretion of S100A8 and S100A9 mRNA were determined. S100A8 and S100A9 mRNA levels were significantly lower in neutrophils compared with macrophages, as expected.

LPS plus IFNγ was used to address the expression changes of S100A9 in macrophages and DCs subjected to inflammatory stimuli. In macrophages, LPS/IFNγ had no effect on S100A8 and S100A9 mRNA (Figure 6D-E).

Significantly increased Mac-2 area (P<0.001), but bone marrow S100A9 deficiency had no effect (P=0.05). D, Mac-2–positive area was quantified in 3 epididymal fat sections/animal. Two-way ANOVA was used to evaluate the effect of diet (chow vs DDC) and genotype (BM_A9+/− versus BM_A9−/− mice) on Mac-2 area (μm²), followed by Bonferroni posthoc analysis. DDC feeding significantly increased Mac-2 area (P<0.001), but bone marrow S100A9 deficiency had no effect (data not shown). Together, these results demonstrate that neither bone marrow S100A9 deficiency nor whole-body S100A9 deficiency affects adipose tissue inflammation, obesity, or diet-induced insulin resistance.
Whole-body S100A8/A9 deficiency does not improve insulin resistance. Whole-body S100A9+/+ (A9+/+) and S100A9−/− (A9−/−) male mice were fed chow or diabetogenic diet with cholesterol (DDC) for 12 weeks. A, Body weights were normalized to starting body weight. Two-way ANOVA was used to evaluate the effect of diet (chow vs DDC) on body weight (percent change from baseline) as a function of time (weeks), followed by Bonferroni post hoc analysis. DDC feeding significantly increased epididymal weight (g), followed by Bonferroni post hoc analysis. DDC-fed A9−/− mice, n = 3; chow-fed A9−/−, n = 3; DDC-fed A9+/+, n = 4; DDC-fed A9−/−, n = 7. B, Epididymal fat pad weight was measured. Two-way ANOVA was used to evaluate the effect of diet (chow vs DDC) on epididymal weight (g), followed by Bonferroni post hoc analysis. DDC feeding significantly increased epididymal weight (P < 0.001), but bone marrow S100A9 deficiency had no effect (P > 0.05). Chow-fed A9−/− mice, n = 3; chow-fed A9−/−, n = 3; DDC-fed A9+/+, n = 4; DDC-fed A9−/−, n = 7. C, Blood glucose (mg/dL), followed by Tukey post hoc test (*P < 0.05, **P < 0.01 for S100A9 mRNA vs S100A9 mRNA at day 0). One-way ANOVA was used to evaluate S100A9 and S100A8 mRNA as a function of time (days), followed by Tukey post hoc test (*P < 0.05, **P < 0.01 for S100A9 mRNA vs S100A9 mRNA at day 0). Results are presented as mean ± SEM or mean ± SEM (A, B, and D).

In atherosclerotic lesions, S100A9 immunoreactivity was frequently present primarily in areas positive for Mac-2 (Figure 6F), a marker of macrophages and DCs (L.B., M.M.A., K.E.B., J.W.H., unpublished observations). The S100A9-positive area also contained occasional cells positive for major histocompatibility complex class II (Figure 6F). Few lesions (5% of lesions analyzed) showed...
occasional cells positive for the neutrophil marker Ly6G. Thus, it is unlikely that neutrophils represent a major source of S100A9 in lesions from LDLR−/− mice. A similar pattern of S100A9 immunoreactivity was apparent in the crown-like structures of inflamed adipose tissue, whereas adipocytes themselves were negative for S100A9 (Figure 6G).

S100A9-Deficient Neutrophils Have a Blunted Inflammatory Response and Show Reduced Accumulation in Inflamed Adipose Tissue

We next asked whether S100A9 deficiency has similar effects in these different myeloid cell populations. S100A9-deficient neutrophils elicited from the peritoneal cavity and then stimulated with LPS released significantly less tumor necrosis factor-α and monocyte chemoattractant protein-1 than wild-type neutrophils (Figure 7A–B). Thus, S100A9 and/or S100A8 has a proinflammatory role in neutrophils.

Neutrophils infiltrate adipose tissue in mice within the first 2 weeks of fat feeding.26 We therefore investigated neutrophil accumulation in epididymal fat in DDC-fed whole-body S100A9+/− and S100A9−/− mice. Myeloperoxidase mRNA was used as a sensitive neutrophil marker. After 14 days of DDC feeding, myeloperoxidase mRNA was detectable in adipose tissue from wild-type mice but not in adipose tissue from S100A9−/− mice (Figure 7C). The number of neutrophils in S100A9+/− mice was low; <1 neutrophil per cross section was identified (data not shown). Conversely, no differences in CD68 mRNA, used as a macrophage and DC marker, were observed between S100A9+/− and S100A9−/− adipose tissue (data not shown). This suggests that S100A9 deficiency inhibits neutrophil infiltration into adipose tissue, corroborating
IL-12p70 release in S100A9 san, another TLR2 ligand, elicited a similarly enhanced effect on DCs compared with wild-type DCs (Figure 8C and 8D). Zymo-

significantly higher release of IL-6 from S100A9-deficient Pam3CSK4, TLR4 and TLR2/1 agonists, respectively, induced 

lated with 250 ng/mL lipopolysaccharide (LPS) overnight. TNF-

H9253

shown), yet in both wild-type and S100A9 

proliferation in the presence of unstimulated DCs (data not 

recombinant S100A8 and S100A9 did not affect T-cell 

with wild-type DCs (Figure 8E). Addition of exogenous 

activated by LPS and IFN 

In contrast to neutrophils, S100A9 

TLR4 Ligands

Inflammatory Response to TLR2 and 

S100A9-Deficient DCs Display an Enhanced 

Inflammatory Response to TLR2 and 

Discussion

We demonstrate that despite the majority of S100A9 being 

derived from circulating myeloid cells, bone marrow–
specific S100A9 deficiency is not sufficient to reduce 

systemic inflammation, insulin resistance, or atherosclero-
sis in fat-fed LDLR 

mice. We propose that the lack of a net effect of S100A9 deficiency in bone marrow–derived 
cells is explained by our findings that 3 different myeloid 
cell populations—neutrophils, macrophages, and DCs—
respond in distinctly different ways after inflammatory 

activation in the setting of S100A9 deficiency. We further 

propose that the relative involvement of distinct myeloid 
cell populations is likely to explain the inconsistent effects 
of S100A9 deficiency in inflammatory diseases.2,18,27 A 

limitation of our study is that myeloid cell populations 

from adipose tissue and the artery wall of insulin-resistant 

hyperlipidemic mice were not evaluated because of the 
difficulty of obtaining large enough quantities of well-

characterized myeloid cell populations from tissues. Such 

studies would provide further insight into the role of 

S100A9 in vivo. Another limitation is that small signifi-
cant differences might have been detected with larger 
groups of mice.

S100A8 and S100A9 play important roles in neutrophil 
migration11 and NADPH oxidase activation.28 S100A9 
deficiency inhibits inflammation in models of sepsis and 

pancreatitis.6–8 These disorders are partially mediated by 

neutrophils, consistent with our present findings, which 
demonstrate reduced neutrophils in inflamed adipose tis-
sue of fat-fed S100A9-deficient mice. Together, these 

findings suggest that S100A9 deficiency reduces 

neutrophil-mediated inflammation. Conversely, S100A9 
inhibits DC maturation,18 and our data suggest that 

S100A9 deficiency increases the inflammatory response of 

DCs. We propose that S100A9 deficiency in diseases in 

which DCs play a more prominent role will result in 
increased tissue inflammation.

S100A8/A9 have been shown to predict cardiovascular 
events1–29 and are highly expressed in rupture-prone le-

cantly reduced T-cell proliferation (Figure 8F). Thus, 

S100A8 and S100A9 have inhibitory functions in activated 

DCs, an effect due in part to secreted S100A8/A9 acting in 
an autocrine fashion.

The inflammatory response of S100A9-deficient macro-

phages was also investigated. Neither bone marrow–
derived macrophages nor thioglycollate-elicited S100A9-
deficient peritoneal macrophages showed a difference in 

LPS-induced proinflammatory gene expression; in tumor 
necrosis factor-α, IL-12p40, or monocyte chemotactant 

protein-1 release; or in zymosan-induced IL-12p40 release 

compared with S100A9 

macrophages (Figure VIIB 

through VIIID in the online-only Data Supplement and data 

not shown).

Thus, DCs from S100A9-deficient mice exhibit an 
increased proinflammatory response and an increased ability to 

induce T-cell proliferation, whereas S100A9 deficiency in 

macrophages has no effect on their inflammatory response to 

TLR ligands.

S100A9-Deficient DCs Display an Enhanced 

Inflammatory Response to TLR2 and 

TLR4 Ligands

In contrast to neutrophils, S100A9 

bone marrow–

derived DCs stimulated with LPS plus IFNγ exhibited 
significant increases in IL-12p40 secretion and iNOS 
mRNA levels (Figure 8A and 8B), 2 markers of inflam-

matory status, compared with S100A9 

DCs. The enhanced proinflammatory response of S100A9 

DCs was confirmed in bone marrow DCs purified by CD11c+ 

selection. In the CD11c+ DC population, LPS and 

Pam3CSK4, TLR4 and TLR2/1 agonists, respectively, induced 
a significantly higher release of IL-6 from S100A9-deficient 

DCs compared with wild-type DCs (Figure 8C and 8D). Zymo-

san, another TLR2 ligand, elicited a similarly enhanced effect on 

IL-12p70 release in S100A9 

DCs (Figure VIIA in the 
online-only Data Supplement).

Furthermore, S100A9 

cells activated with LPS plus 

IFNγ significantly increased T-cell proliferation compared with 

wild-type DCs (Figure 8E). Addition of exogenous 

recombinant S100A8 and S100A9 did not affect T-cell 

proliferation in the presence of unstimulated DCs (data not 

shown), yet in both wild-type and S100A9 

DCs activ-

by LPS and IFNγ, exogenous S100A8/A9 signifi-

Figure 7. S100A9-deficient neutrophils secrete less tumor necrosis factor-α (TNF-α) and monocyte chemotactant protein-1 (MCP-1). Thioligolate-elicited peritoneal neutrophils were obtained from S100A9+/+ and S100A9−/− mice and stimu-
lated with 250 ng/mL lipopolysaccharide (LPS) overnight. TNF-α (A) and MCP-1 (B) were measured in con-
trolled media by ELISA. The results are presented as mean±SEM (A and B). Statistical 
analysis was performed by 1-way ANOVA for TNF-α and 
MCP-1 secretion (pg/mg protein), followed by Tukey post hoc 
test. A9+/+ and A9−/− control vs A9+/+ LPS: ***P<0.001; A9+/+ 
LPS vs A9−/− LPS: ***P<0.001; other comparisons: P>0.05. 
n=3 except for LPS-stimulated cells in A (n=4). C, Epididymal 
adipose tissue harvested from 3 S100A9+/+ and S100A9−/− 
mice after a 2-week regimen on diabetogenic diet with choles-
terol. Levels of myeloperoxidase (MPO) mRNA were evaluated 
by reverse transcriptase polymerase chain reaction in each 
mouse.

evidence that S100A9 plays an important role in promoting 

neutrophil migration and their inflammatory status.
ApoE whole-body S100A9 deficiency inhibits atherosclerosis in role in atherosclerosis. However, we show that LDLR eloid cells.19,20 S100A8/A9 can induce chemotactic factor reducible expression of S100A8 and/or S100A9 in nonmyeloid cell–derived S100A8/A9 may also act in a paracrine fashion on artery wall cells. In addition, ApoE given that S100A8/A9 have extracellular actions,6,14–15,32 myeloid cell composition between ApoE−/− and LDLR−/− mice.

Conclusions
S100A8/A9 differentially modulate the inflammatory responses of myeloid cells in diametrically opposed manners. S100A8/A9 are proinflammatory in neutrophils, have no detectable inflammatory effects in macrophages, and are antiinflammatory in DCs. The main source of S100A9 in lesions of atherosclerosis, at least in LDLR−/− mice, appears to be early macrophages or DCs. Further study is needed to fully understand the functions of S100A8/A9 in specific cell populations and disease states before S100A8/A9 are considered therapeutic targets.

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Disclosures

None.

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**CLINICAL PERSPECTIVE**

It has previously been demonstrated that elevated plasma levels of S100A9 (also known as myeloid related protein-14) in complex with its binding partner S100A8 (myeloid related protein-8) predict increased risk of future cardiovascular events in healthy postmenopausal women and recurrent events in patients with acute coronary syndromes. Furthermore, apolipoprotein E–deficient mice that are also deficient in S100A9 exhibit reduced atherosclerosis. These important findings suggest that S100A9 is both a biomarker and a mediator of atherosclerosis and cardiovascular events. Most of the constitutively secreted S100A9 is believed to be derived from myeloid cells. We demonstrate that low-density lipoprotein receptor–deficient mice that lack S100A9 in bone marrow–derived cells, including myeloid cells, are not protected against diet-induced atherosclerosis or insulin resistance. Furthermore, S100A9 deficiency differentially modifies phenotypic states of myeloid cell populations. S100A9-deficient neutrophils exhibit a reduced secretion of cytokines, whereas S100A9-deficient dendritic cells show an exacerbated release of cytokines. The effect of S100A9 deficiency on atherosclerosis and other inflammatory diseases is therefore predicted to depend on the relative contribution of these cell types at different stages of disease progression. Furthermore, S100A9 expression in nonmyeloid cells is likely to contribute to atherosclerosis. Further study is needed to fully understand the functions of S100A8/A9 in specific cell populations and disease states before S100A8 and S100A9 are considered therapeutic targets.
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Supplemental Methods

Isolation and culture of thioglycollate-elicited cells and bone marrow-derived cells

Thioglycollate-elicited neutrophils and macrophages were isolated from C57BL/6J mice, S100A9+/+ mice, or S100A9−/− mice, as described previously1. In short, 2 mL thioglycollate (40 mg/mL) was injected intraperitoneally. The ascites was collected by sterile lavage. Neutrophils were collected 4 h after thioglycollate injection followed by a 1 h adherence purification before collecting non-adherent cells. The macrophages were collected five days post thioglycollate injection and adherence-purified for 1 h, followed by a wash with PBS to remove non-adherent cells.

Bone marrow was harvested from C57BL/6J, S100A9+/+ and S100A9−/− mice, and 2x10⁶ cells/well were plated in 6-well plates in RPMI containing 11.2 mmol/L D-glucose, and were cultured for 5-7 days. The concentration of glucose (~200 mg/dL) was similar to that observed in DDC-fed insulin resistant mice. Macrophages were generated using 30% L-conditioned medium, as a source of M-CSF, for 5-7 days, and DCs were generated using 3-10 ng/mL GM-CSF for 5-7 days. Monocyte enrichment from bone marrow was performed using a negative selection monocyte enrichment kit (Easy Sep, Vancouver, BC, Canada). 4x10⁵ monocyte-enriched bone marrow cells were plated per well in 24-well plates, and cultured in 30% L-conditioned medium for 0-8 days. To classically activate macrophages and DCs, cells cultured for 5 days were stimulated with 5 ng/mL LPS plus 12 ng/mL IFNγ during the last 24 h. To examine TLR responses, DCs cultured for 7 days were purified using CD11c+ beads and separated on a magnetic activated cells sorter (Miltenyi Biotec Inc. CA). 5x10⁴ purified cells were plated per well in 96-well plates and exposed to varying concentrations of the TLR agonists zymosan, LPS, CpG-B, and Pam3CSK4 overnight before conditioned media were collected.

T-cell isolation and allogeneic mixed lymphocyte reaction

T-cells from single-cell suspensions of spleen and peripheral lymph nodes obtained from BALB/c mice were generated and purified as described by Pendl et al.2, by nylon wool non-adherence and subsequent immunomagnetic depletion of contaminating cells. Immunomagnetic depletion of contaminating cells was done using monoclonal antibodies against CD45R/B220 (clone RA3-6B2), CD11b (clone M1/70), CD16/32 (clone 2.4G2), GR-1/Ly6G (clone RB6-8C5), CD24 (clone M1/69), T cells (clone GL3), and NK-T cells (clone U5A2-13; all from BD Pharmingen), followed by incubation with goat anti-rat IgG MicroBeads (Miltenyi Biotec, Bergisch Gladbach, Germany). The antibody-negative cell fraction was separated with an automated magnetic cell sorter (autoMACS, Miltenyi Biotec).

Mixed lymphocyte reactions (MLR) were performed as described by Labeur et al.3 with generous assistance of Dr. Thomas F. Scholzen (University of Muenster, Muenster, Germany). Briefly, DCs were incubated in graded concentrations together with 2x10⁵ T-cells per well in 96-well plates. The primary MLR was performed in RPMI 1640 supplemented with 10% FBS, 100 U/mL penicillin, 100 mg/mL streptomycin,
0.1 M nonessential amino acids 2 mmol/L L-glutamin, 1 mmol/L sodium pyruvate, 0.01 M HEPES, 50 µmol/L 2-mercaptoethanol, and 20 µg/mL gentamycin. DCs were matured with 100 ng/mL LPS with 10 ng/mL IFNγ for 24 h before washed and added to 2x10⁵ T-cells at a starting ratio of 20:1 and incubated for 96 h in 96-well round-bottom microtiter plates. T-cell proliferation was measured by adding 1 µCi [³H]- thymidine to the cultures and subsequent liquid scintillation counting using an automated liquid scintillation detector (Microbeta Workstation, Wallac) after an overnight incubation period (16 h).

*Generation of recombinant mouse S100A9 and S100A8*

Both mS100A9 and mS100A8 cDNAs were used as templates to generate PCR-fragments with suitable cloning ends. The PCR fragments were purified and cloned into pQE32 (Qiagen) according to standard protocols. Individual clones were analyzed by PCR and SDS/PAGE. Positive clones were grown to D(580 nm) = 0.6 and subsequently induced by 1 mmol/L isopropyl-1-beta-D-galactopyranoside for 2 h. Cell pellets were resuspended in NaCl/Tris, sonicated, frozen and stored at -20°C. His-tagged proteins were purified using the TALON (Clontech) affinity matrix. Briefly, cell pellets were resuspended in NaCl/Tris/8 mol/L urea. Insoluble material was removed by centrifugation and the supernatant was incubated with the affinity matrix. Proteins were renatured on the column by washing the column with buffers containing decreasing concentrations of urea. Finally, recombinant proteins were eluted with NaCl/Tris/300 mmol/L imidazole. Purity of the recombinant proteins was demonstrated by SDS/PAGE.

*mRNA analysis*

Quantitative real-time PCR was performed with the primers listed in Supplementary Table 1. Total RNA was isolated from cells using Qiagen RNeasy® Mini Kits (Qiagen, Valencia, CA) followed by DNase treatment. Epididymal fat was homogenized in Qiazol and extracted by using the Qiagen RNeasy® Lipid Tissue Mini Kit (Qiagen, Valencia CA) and then subjected to DNase treatment. Real-time PCR was conducted in 2 steps. mRNA was first reverse transcribed (RT) using 20 µL of RT reaction mixture (0.1-1 µg template RNA, 1.0 unit of Fermentas M-MuLV RT, 0.5 unit of RiboLock RNase Inhibitor, 100 pmol/L random hexamers, 1 mmol/L dNTPs) at 25°C for 10 min, 42°C for 60 min, and the reaction was terminated at 70°C for 10 min. The resulting cDNA was diluted and qPCR was performed in a 20 µL reaction using 10 ng cDNA, SYBR® Green PCR Master Mix (Fermentas, Glen Burnie, MD) and 400 nmol/L of each primer. RT-PCR was run on a 7900HT PCR system (Applied Biosystems, Calsbad, CA) for 40 cycles at 95°C for 15 seconds, 60°C for 1 min. Resulting Ct values were calculated using the ΔΔCt method to express values as fold over control samples. All samples were run in at least duplicates and statistical analysis was performed on 2- (ΔΔCt) values. Primers were from Invitrogen, and were designed using Primer-BLAST (NCBI, Bethesda, MD). Semi-quantitative PCR was used to evaluate the presence or absence of MPO mRNA in adipose tissue and S100A8 and S100A9 mRNA in circulating leukocytes from S100A9⁺⁻/ and S100A9⁻/⁻ mice. Briefly, 10-20 ng cDNA was amplified
using Taq Polymerase (0.5 U, Promega, Madison WI), 1 mmol/L dNTPs, 400 nM each primers, in 20 µl buffer. Samples were run for 40 cycles in a PCR system 9700 (Applied Biosystems, Foster City, CA).

**Western blots, ELISAs, mass spectrometry, and flow cytometry**

Secretion of IL-12p40, IL-12p70, IL-6, MCP-1, and TNF-α was quantified by ELISA (eBioscience). For analysis of S100A8/A9 cellular protein levels, total cell lysates (10-50 µg) were loaded onto 15% SDS-PAGE gels, separated and transferred onto 0.2 µm pore size PVDF membranes (Millipore Immobilon P; Billerica, MA). Detection was accomplished by using the following primary antibodies: S100A8 antibody (0.2 µg/mL), S100A9 antibody (0.2 µg/mL, both from RnD systems Minneapolis, MN), and β-actin antibody (mouse monoclonal; 1:10,000 dilution; Sigma-Aldrich). Horseradish peroxidase-conjugated secondary antibodies were used.

The secreted proteome form macrophages, macrophages activated with LPS plus IFN-γ, and DCs was collected for mass spectrometric analysis as previously described. Peptides unique to S100A8 and S100A9 were quantified by spectral counting.

Flow cytometry was conducted on bone marrow-derived macrophages and DCs. Cells were detached from culture plates using a phosphate buffered solution of 8 mg/mL lidocaine with 5 mmol/L EDTA. Cell suspensions were incubated with the following antibodies (eBioscience) for 30 min at 4°C: MHC class II-APC (I-A/E, 0.025 µg/million cells), and CD11c-PE (0.15 µg/million cells). Cells were run on a on a FACS Canto (BD Biosciences, San Jose, CA) and all analyses were based after a live cell gate was established. To estimate relative expression, statistics were performed on geometric means.

**Bone marrow transplants and analysis of atherosclerosis and insulin resistance**

In the 12-week insulin resistance study, male whole-body S100A9+/+ and S100A9−/− mice (10-12 weeks old) were either kept on a chow diet or switched DDC (BioServ No.F4997). The mice were kept on the diet for 12 weeks, during which body weights were monitored weekly. Insulin resistance was evaluated by conducting glucose tolerance and insulin tolerance tests at 10 and 12 weeks, respectively. A glucose tolerance test (GTT) was performed by injecting 1.5 g/kg dextrose into 5 h fasted mice and blood glucose was measured at 0, 15, 30, 60, and 120 min after injection. An insulin tolerance test (ITT) was performed by injecting 0.7 U/kg insulin into 5 h fasted mice and measuring blood glucose at the same indicated times. Area under the curve was calculated and used to determine difference between groups. At the end of 12 weeks, mice were saline perfused and epididymal fat was dissected, weighed, and fixed in 4% paraformaldehyde, 5% sucrose, 20 µmol/L EDTA, pH 7.4.

Similar 12-week studies were performed in chow-fed and DDC-fed male LDLR−/− (S100A9+/+) mice. At week 11, GTTs were performed, and thioglycollate-elicited peritoneal macrophages were harvested at the end of the 12-week study.
In the 24-week bone marrow transplant study, male LDLR-deficient mice (12-14 weeks old) received intravenous bone marrow transplants from S100A9+/+ or S100A9−/− mice (5x10⁶ cells; purified of erythrocytes) following lethal irradiation (10 Gy). The mice recovered for 3 weeks, and were then switched to either chow or DDC for an additional 24 weeks. Body weights were measured weekly, and blood glucose (Precision Q·I·D Complete Blood Glucose Monitoring System; MediSense Inc.) and blood cholesterol (CardioChek; Polymer Technology Systems Inc.) were determined using blood from the saphenous vein at the beginning and end of the study. There were no differences in body weight, blood glucose, or cholesterol between the groups at baseline. Insulin resistance was assessed using the GTT and ITT after 20 and 22 weeks respectively, following the procedure described previously with the exception of using 1.2 U insulin/kg for ITT. At the end of the study, mice were perfused under physiological pressure using saline. Epididymal and inguinal fat pads were dissected and weighed, and the epididymal fat was fixed in 4% paraformaldehyde, and then sectioned for immunohistochemical analysis. The aortic sinus and brachiocephalic artery (BCA) were dissected, fixed, embedded, and serial sectioned. Every 4th section was stained using a Movat’s pentachrome stain procedure, as described previously. Adjacent sections were stained using antibodies described below. Maximal lesion area was determined by an investigator in a masked fashion. Plasma IL-6 was analyzed using ELISA (RnD Systems), and triglycerides were determined using a colorimetric assay from Sigma Aldrich.

**Immunohistochemistry**

Selected tissue sections from epididymal adipose tissue, aortic sinus, and BCA were rehydrated and endogenous peroxidase activity was blocked using 3% hydrogen peroxide. Antigen retrieval using microwave heating in citrate buffer (pH 6.0, 0.01 mol/L) for 30 min from the start of boiling prior to blocking with hydrogen peroxide was conducted for S100A9 staining. Antigen retrieval using proteinase K (20 µg/mL) for 15 min at 37°C was used for Ly6G stains. Successful antigen retrieval for CD3 stains was achieved by using heat-induced epitope retrieval with DAKO Target Solution, Tris-EDTA, pH 9.0, for 5 minutes. Other antibodies did not require antigen retrieval. The sections were then incubated 4°C overnight with the following primary antibodies in 3% goat serum except anti-S100A9, which was diluted in the presence of 3% rabbit serum: Anti-S100A8 and anti-S100A9 (0.2 µg/mL, RnD Systems), anti-Mac-2 (1 µg/mL, Cedarlane), anti-Ly6G (1 µg/mL, BD Pharmingen), anti-MHC class II (1 µg/mL, eBiosciences). Relevant isotype controls were used to confirm specificity of the immunoreactivity, including Rat IgG2a (Cedarlane), Rat IgG2b (eBioscience), and Goat IgG (Zymed). A rat anti-human CD3 antibody (AbD Serotec; MCA1477) at 1:100 dilution was used to detect T-cells in atherosclerotic lesions. Spleen was used as a positive control. Sections were washed and incubated with biotinylated goat anti-rat (Southern Biotech) or rabbit anti-goat (Vector) secondary antibodies. The slides were then washed and incubated with streptavidin-HRP complex and developed with 3,3′-diaminobenzidine.
Supplemental references


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<th>Supplemental Table 1. RT-PCR Primers</th>
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Supplemental Figure 1. S100A9 is similarly expressed in LPS/IFNγ-stimulated macrophages, and S100A8 is not increased in S100A9-deficient cells under basal conditions of following LPS/IFNγ-stimulation. Bone marrow cells from S100A9+/+ and S100A9−/− mice were cultured in the presence of M-CSF for 5 days prior to analysis. S100A8 and S100A9 protein levels were analyzed by Western blot. A representative Western blot is shown. The experiments were repeated several times with similar results.
Supplemental Figure 2. Bone marrow S100A9-deficiency does not affect atherosclerosis in the BCA. Male LDLR−/− mice were transplanted with wild type (BM_A9+/+) or S100A9-deficient (BM_A9−/−) bone marrow, and fed chow or DDC for 24 weeks. (A) Total atherosclerotic lesion area was quantified in the BCA based on Movat’s pentachrome-stained sections, using computer-assisted imaging analysis. The results are expressed as mean + SEM (N=10 chow-fed BM_A9+/+; N=6 chow-fed BM_A9−/−; N=12 DDC-fed BM_A9+/+; N=9 DDC-fed BM_A9−/− mice). Statistical analysis was performed using two-way ANOVA to evaluate the effect of diet (chow versus DDC) and genotype (BM_A9+/+ versus BM_A9−/− mice) on the BCA lesion area (µm²), followed by Bonferroni post-hoc analysis to compare the four different groups. DDC-feeding significantly increased lesion area (p<0.05), but bone marrow S100A9-deficiency had no effect (p>0.05). (B) Anti-Mac2 was used as a Mac/DC marker, and positive staining was quantified at the maximal site of the BCA lesion for each mouse. The results are expressed as mean + SEM (N=10 chow-fed BM_A9+/+; N=7 chow-fed BM_A9−/−; N=11 DDC-fed BM_A9+/+; N=9 DDC-fed BM_A9−/− mice). Statistical analysis was performed using two-way ANOVA to evaluate the effect of diet (chow versus DDC) and genotype (BM_A9+/+ versus BM_A9−/− mice) on the Mac-2-positive lesion area (µm²), followed by Bonferroni post-hoc analysis to compare the four different groups. DDC-feeding significantly increased Mac-2 area (p<0.001), but bone marrow S100A9-deficiency had no effect (p>0.05).
Supplemental Figure 3. Cells positive for S100A9, Mac-2, and Ly6G are co-localized in BCA lesions. BCA cross-sections from a male LDLR<sup>−/−</sup> mice transplanted with wild type (BM_A9<sup>+/+</sup>) bone marrow, and fed DDC for 24 weeks. Lesion morphology was determined from Movat’s pentachrome-stained sections, and adjacent sections were stained with anti-Mac-2, anti-S100A9, and anti-Ly6G antibodies. Representative Ly6G-positive neutrophils in the lesion and blood clot are indicated by arrows. Bar, 100 µm
Supplemental Figure 4. TNF-α stimulates increased S100A8 mRNA in mouse endothelial cells. Mouse C57BL/6 endothelial cells were isolated from heart, using a cell sorting method modified from Ieronimakis et al. (PLoS One. 2008;3:e0001753). Endothelial cells were used at passages 3-10 and stimulated with 20 ng/mL TNF-α for 24 h. The results are expressed as mean + SEM (N=3). Statistical analysis was performed using one-way ANOVA to evaluate the effect of TNF-α on S100A8 and S100A9 mRNA levels (fold over control), followed by Tukey post-hoc analysis. TNF-α significantly elevated S100A8 mRNA levels (*p<0.05), but had no significant effect on S100A9 mRNA levels (p>0.05) compared to controls.
Supplemental Figure 5. CD11c and MHC class II expression is increased in cells differentiated in the presence of GM-CSF compared to M-CSF.

Bone marrow cells were cultured in 30% L-conditioned medium (M, Green) or 3 ng/mL recombinant mouse GM-CSF (DC, Blue) for 5 days. The cells were then stained with PE-anti-CD11c and APC-anti-I-A/I-E (MHC class II) or the matching isotype controls (Red) before analyzed on a flow cytometer. CD11c (A) and MHC class II (B) was measured on all live cells. Cell surface expression of CD11c (C) and MHC class II (D) was quantified using the geometric mean. The results in C-D are expressed as means + SEM. Statistical analysis was performed using a two-tailed Student’s t-test to compare cell surface CD11c geometric means of macrophage and dendritic cell populations (C; N=3/group; *p<0.05) or cell surface MHC class II geometric means (D; N=3/group; **p<0.01). Mac or M, macrophages differentiated in the presence of M-CSF; DC or D, dendritic cells differentiated in the presence of GM-CSF; N or Isotype, negative control.
Supplemental Figure 6. LPS/IFNγ-stimulation does not significantly alter S100A9 secretion from DCs. Bone marrow cells from three different mice were cultured in the presence of 3 ng/mL recombinant mouse GM-CSF for 5 days, and were then incubated with LPS (5 ng/mL) and IFNγ (12 ng/mL) for 24 h (DC + LPS/IFNγ) or were left as controls (DC). Conditioned media were collected and analyzed for S100A9 levels by Western blots. The samples were normalized to the number of cells and volume.
Supplemental Figure 7. DDC feeding does not significantly alter S100A8 or S100A8 mRNA levels in peritoneal macrophages from hypercholesterolemic insulin resistant LDLR⁻/⁻ mice. Male LDLR⁻/⁻ mice were fed regular chow or DDC for 12 weeks. DDC-fed mice had blood cholesterol levels of 1,039 ± 181 mg/dL as compared to 332 ± 23 mg/dL in chow-fed mice (p<0.01 by unpaired Student’s t-test). DDC-fed mice were also insulin resistant, with a GTT area-under-the-curve of 1,963 ± 50 (mg/dL)min as compared to 1,328 ± 76 (mg/dL)min in chow-fed mice (p<0.001 by unpaired Student’s t-test). The results are expressed as mean ± SEM (N=5/group). Statistical analysis was performed using one-way ANOVA to evaluate the effect of diet (chow/control versus DDC) on S100A8 and S100A9 mRNA levels (fold of control), followed by Tukey posthoc test. There were no significant differences (p>0.05).
Supplemental Figure 8. S100A9-deficient DCs exhibit an increased inflammatory response to TLR2 activation (A), but S100A9-deficiency in macrophages does not affect their response to TLR stimulation (B-D). DCs were obtained by isolation of CD11c⁺ bone marrow cells following differentiation in the presence of GM-CSF. 5×10⁴ cells per well in 96-well plates were stimulated with the indicated concentrations of a TLR2 agonist (zymosan; A). Secretion of IL-12p70, a major Th1 immune cytokine, was measured by ELISA. Thioglycollate-elicited macrophages were stimulated with 250 ng/mL LPS (B-D) or 25 µg/mL zymosan (C). Secreted TNF-α (B), IL-12p40 (C) and MCP-1 (D) was measured by ELISA and normalized to cellular protein. Results are expressed as means ± or + SEM. Two-way ANOVA was used to compare the effects of S100A9-deficiency on the parameters and units indicated by the y-axes, followed by Bonferroni post-hoc analysis; N=3/group for all groups except controls in (B), for which N=4. In (A), increasing zymosan concentration resulted in a significant (p<0.05) stimulation of IL-12p70 secretion, and S100A9-deficiency enhanced IL-12p70 secretion (*p<0.05). The Bonferroni posthoc test revealed that the difference between S100A9⁺/+ and S100A9⁻/- cells was significant (**p<0.01) at 25 µg/ml zymosan. In (B-D), LPS/zymosan treatment increased cytokine secretion (p<0.001 in B; p<0.01 in C; p<0.001 in D) with no effect between S100A9⁺/+ and S100A9⁻/- cells. A9⁺/+; S100A9⁺/+ cells; A9⁻/-; S100A9⁻/- cells