E-Selectin-Dependent Inflammation and Lipolysis in Adipose Tissue Exacerbate Steatosis-to-NASH Progression via S100A8/9

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SUMMARY
E-selectin, a key adhesion molecule for neutrophils, is highly up-regulated in adipose tissue of nonalcoholic steatohepatitis patients compared with individuals with fatty liver. Deletion of the E-selectin gene or inhibition of neutrophil-derived S100A9 in mice ameliorated adipose tissue inflammation, improving nonalcoholic steatohepatitis.

BACKGROUND & AIMS: Nonalcoholic steatohepatitis (NASH) is a leading cause of chronic liver disease, characterized by steatosis and hallmark liver neutrophil infiltration. NASH also is associated with adipose tissue inflammation, but the role of adipose tissue inflammation in NASH pathogenesis remains obscure. The aim of this study was to investigate the interplay between neutrophil recruitment in adipose tissue and the progression of NASH.

METHODS: A mouse model of NASH was obtained by high-fat diet (HFD) feeding plus adenovirus-Cxcl1 overexpression (HFD + AdCxcl1). Genetic deletion of E-selectin (Sele) and treatment with an S100A9 inhibitor (Paquinimod) were investigated using this model.

RESULTS: By analyzing transcriptomic data sets of adipose tissue from NASH patients, we found that E-selectin, a key adhesion molecule for neutrophils, is the highest up-regulated gene among neutrophil recruitment-related factors in adipose tissue of NASH patients compared with those in patients with simple steatosis. A marked up-regulation of Sele in adipose tissue also was observed in HFD + AdCxcl1 mice. The HFD + AdCxcl1-induced NASH phenotype was ameliorated in Sele knockout mice and was accompanied by reduced lipolysis and inflammation in adipose tissue, which resulted in decreased serum free fatty acids and proinflammatory adipokines. S100A8/A9, a major proinflammatory protein secreted by neutrophils, was highly increased in adipose tissue of HFD + AdCxcl1 mice. This increase was blunted in the Sele knockout mice. Therapeutically, treatment with the S100A9 inhibitor Paquinimod reduced lipolysis, inflammation, and adipokine production, ameliorating the NASH phenotype in mice.

CONCLUSIONS: E-selectin plays an important role in inducing neutrophil recruitment in adipose tissue, which subsequently promotes inflammation and lipolysis via the production of S100A8/A9, thereby exacerbating the steatosis-to-NASH progression. Targeting adipose tissue inflammation therefore may represent a potential novel therapy for treatment of NASH. (Cell Mol Gastroenterol Hepatol 2022;13:151–171; https://doi.org/10.1016/j.jcmgh.2021.08.002)
Nonalcoholic fatty liver disease (NAFLD) encompasses a series of chronic liver disorders with varying injury severity ranging from simple steatosis to nonalcoholic steatohepatitis (NASH), cirrhosis, and hepatocellular carcinoma (HCC). NASH is characterized by hepatic steatosis, lobular inflammation, hepatocyte damage, and, typically, also pericellular fibrosis. NASH can progress further into cirrhosis and eventually to primary hepatocellular carcinoma, posing a serious health risk. It is estimated that NASH affects 1.5%–6.5% of the general population, and that figure is expected to dramatically increase in the next decade. The mechanisms involved in the transition of simple steatosis, which is mostly asymptomatic, toward NASH have not been fully elucidated. Our group previously showed that the hepatic infiltration of neutrophils plays a crucial role in promoting the steatosis-to-NASH transition. However, the contribution of adipose tissue to this so-called neutrophil-driven NASH has not been documented. Because a crosstalk between adipose tissue inflammation and the liver was suggested to be implicated in NASH development, we investigated in this study how neutrophil recruitment in adipose tissue may influence NASH.

Immune cell recruitment requires vascular endothelial cell activation and expression of adhesion molecules necessary for leukocyte extravasation from the vasculature to areas of inflammation. As per the leukocyte adhesion cascade paradigm (consisting of capture, rolling, arrest, adhesion, and transendothelial migration), the rolling of leukocytes is mediated by selectins. Therefore the regulated expression of selectins and their ligands initiate an inflammatory response. The selectin family consists of 3 cell adhesion molecules, namely L-selectin (SELL), which is expressed by most leukocytes, and E-selectin (SELE) and P-selectin (SELP), which are expressed by activated endothelial cells and interact with glycosylated ligands such as CD44 and SELP glycoprotein ligand 1.

We previously showed that chronic-binge ethanol feeding in mice up-regulates the hepatic expression of "Sele", inducing hepatic neutrophil accumulation, injury, and inflammation. Deletion of the "Sele" gene reverted this process, preventing hepatic neutrophil infiltration and reducing alcohol-induced liver injury. This made us believe that E-selectin might also play a role in promoting the development of steatosis and NASH. We found that "SELE/Sele" messenger RNAs (mRNAs) indeed were up-regulated in human and mouse livers during NASH, compared with normal livers or steatotic livers. However, this up-regulation was much more pronounced in visceral adipose tissues than in liver tissues, which allowed us to hypothesize that E-selectin involvement in NASH progression was not exclusively in the liver, but also, and even predominantly, in adipose tissue. In the current study, we found that deletion of the "Sele" gene also reduced liver inflammation and injury in a mouse model of NASH induced by high-fat diet feeding plus adenovirus-Cxcl1 overexpression. Interestingly, deletion of the "Sele" gene markedly reduced adipose tissue neutrophil recruitment and resulted in a decrease in lipolysis and secretion of free fatty acids (FFAs) and proinflammatory adipokines. Furthermore, we found that expression of "S100A8" and "S100A9" (also known as myeloid-related protein [MRP] 8 and MRP14, respectively), which are Ca\(^{2+}\)-binding proteins and represent 40% of neutrophil cytosolic protein weight, is highly increased in adipose tissue in HFD-induced NASH mice. The "S100A8/A9" heterodimer released by activated neutrophils has been described as an innate immune mediator during the pathogenesis of multiple diseases, and higher expression of "S100A8" and "S100A9" have been documented in visceral adipose tissue of type 2 diabetic obese patients. However, the role of "S100A8/A9" in steatosis-to-NASH progression has not been documented, although increased serum "S100A8/A9" levels in NASH patients have been reported. In the current study, we found that an increase of "S100A8" and "S100A9" proteins in adipose tissue is dependent on E-selectin because this increase was reduced markedly in "Sele" knockout (KO) mice. Finally, administration of Paquinimod (PAQ), a small molecule that inhibits"S100A9" by interacting with its corresponding receptors (ie, receptor for advanced glycation end products and Toll-like receptor 4), significantly reduced adipose tissue lipolysis and liver injury in mice under NASH conditions, suggesting that inhibition of "S100A9" proteins may represent a potential novel therapy against NASH.

**Results**

E-Selectin in Adipose Tissue Is Highly Up-regulated in Patients and Mice With NASH

Analysis of transcriptomic data sets of adipose tissue from NASH patients showed that among the neutrophil-recruitment genes, "E-selectin (SELE)" was the highest up-regulated gene in the adipose tissue of NASH, compared with adipose tissue from patients with steatosis or healthy livers (Figure 1A). The expression of other selectin molecules in the same cohorts was not (such as: "SELL") or was only slightly (such as: "SELL") increased during NASH. Furthermore, the induction of "VCAM1 ICAM1", which are adhesion molecules that play a role in later stages of the pathogenic process involved in NASH, was significantly lower in adipose tissue from patients with NASH compared with healthy controls. These findings suggest that E-selectin may play a crucial role in the pathogenesis of NASH, potentially by mediating neutrophil recruitment in adipose tissue and contributing to the development of liver injury.

**Abbreviations used in this paper**: AdCxcl1, adenovirus-Cxcl1; ALT, alanine aminotransferase; ANGPTL3, angiopoietin-like protein 3; AST, aspartate aminotransferase; CXCL1, C-X-C motif chemokine ligand 1; ELISA, enzyme-linked immunosorbent assay; FFA, free fatty acid; GGT, gamma-glutamyltransferase; HFD, high-fat diet; HOMA-IR, homeostatic model assessment of insulin resistance; IL, interleukin; ITT, insulin tolerance test; KO, knockout; MPO, myeloperoxidase; mRNA, messenger RNA; MRP, myeloid-related protein; NAFLD, nonalcoholic fatty liver disease; NASH, nonalcoholic steatohepatitis; NA, normal adipose tissue; PP, primiparous; P-selectin; TSEC, immortalized liver sinusoidal endothelial cell line; WT, wild-type.
Figure 1. E-selectin is predominantly up-regulated in adipose tissue (AT) of patients and mice with NASH. (A) Normalized gene expression (microarray data sets obtained from Du Plessis et al. and Lake et al.) in visceral adipose tissue and liver (L) of patients suffering from steatosis (n_{AT} = 6, n_{L} = 10) and NASH (n_{AT} = 13, n_{L} = 15) compared with those from healthy controls (n_{AT} = 8, n_{L} = 19). (B–E) WT mice were subjected to chow diet (Chow) (n = 7), a 3-month high-fat diet plus AdGfp (HFD) (n = 7) to induce steatosis or 3-month HFD plus AdCxcl1 to induce NASH (HFD + AdCxcl1) (n = 7). Adipose and liver tissues were collected and analyzed. (B) RT-qPCR of adipose tissue and liver. (C) Immunofluorescence staining of E-selectin (red), CD31 (green), and 4',6-diamidino-2-phenylindole (DAPI) (blue) of adipose tissue. (D) MPO and F4/80 immunostaining of adipose tissues. (E) RT-qPCR of adipose tissue. *P < .05 for NASH vs normal, **P < .05 for steatosis vs normal or NASH vs normal. #P < .05.
leukocyte adhesion cascade, were induced much less prominently than SEL. A trend in line with the SEL expression also was observed for the up-regulation of CD44, which encodes for a cell-surface glycoprotein that acts as the major E-selectin ligand and for the up-regulation of SELL ligand. Interestingly, the expression of several inflammatory markers, including CCL2, C-X-C motif chemokine ligand 1 (CXCL1), and IL1B, were increased in adipose tissue from NASH patients compared with those from healthy controls or with fatty liver. As illustrated in Figure 1A (right panel), analysis of transcriptomic data sets of human NASH liver samples showed that among the neutrophil-recruitment genes, IL8 was the highest up-regulated gene in liver tissues from NASH, compared with samples from patients with steatosis or healthy livers. Hepatic SEL expression also was higher during NASH compared with steatosis or healthy livers, but not as prominently as in adipose tissue. Furthermore, hepatic expression of VCAM1 and SELL was also up-regulated in the NASH cohort. However, the expression of SEL and ICAM1 did not differ among patient groups. Interestingly, the expression of major inflammatory markers CCL2, CXCL1, IL6, and IL1B was increased in liver tissues from NASH patients compared with those from healthy controls or with fatty liver.

Next, we examined the expression of Sele in a HFD-Induced mouse NASH model and found that Sele expression in the liver and adipose tissue was up-regulated compared with HFD-fed mice injected with a control virus expressing green fluorescence protein (HFD-AdGFP) (simple steatosis model) (Figure 1B). Such up-regulation was seen predominantly in visceral adipose tissue, although the liver also showed a significant Sele increase during NASH, indicating the similarity to human data. E-selectin ligands Cd44 and Selplg also were up-regulated, although the latter did not reach significant levels in adipose tissue (Figure 1B). The increase of E-selectin in adipose tissue also was confirmed by immunofluorescence staining in which endothelial cells, labeled by CD31 (green) showed a higher expression of E-selectin (red) in adipose tissue from HFD-Induced mice than those from HFD-fed mice (Figure 1C).

We previously reported that the number of neutrophils and macrophages was increased markedly in the liver tissues of HFD-Induced mice compared with those from HFD-fed mice. Likewise, we found that adipose tissue from HFD-Induced mice had greater neutrophil and macrophage infiltration than those from HFD-fed mice, as shown by immunostaining of myeloperoxidase (MPO) and F4/80 (Figure 1D), and by reverse-transcription quantitative polymerase chain reaction (RT-qPCR) analysis of the neutrophil marker Ly6g, although no increase in Adgre1 expression, which codes for F4/80, was observed (Figure 1E).

To examine the correlation between Sele expression in activated endothelial cells and neutrophil recruitment, we performed in vitro experiments using an immortalized liver sinusoidal endothelial cell line (TSEC). Treatment with a range of cytokines, including interleukin (IL)1β, tumor necrosis factor α, and transforming growth factor β, as well as cell culture supernatants of primary mouse hepatocytes previously treated with palmitic acid, significantly up-regulated Sele mRNA levels in TSEC (Figure 2A). Palmitic acid treatment alone also up-regulated E-selectin protein levels in TSECs (Figure 2B). Co-culture of activated TSEC cells with freshly isolated neutrophils resulted in increased adhesion of the latter to the TSEC monolayers, indicating a correlation between Sele expression and neutrophil recruitment (Figure 2C and D).

**Deletion of the Sele Gene Slightly But Significantly Reduces Liver Damage Associated With Simple Steatosis in HFD-Fed Mice**

To examine the role of E-selectin in the pathogenesis of nonalcoholic fatty liver disease, we first used E-selectin KO mice (Sele KO) fed a HFD for 3 or 6 months, and found that after 3 months of HFD feeding, Sele KO showed a decreasing trend in serum levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) compared with wild type (WT) mice (Figure 3A). This difference, however, was not statistically significant. Furthermore, no changes in characteristic liver steatosis, body weight, or liver to body weight ratio were observed between WT and Sele KO mice (Figure 3B and C). MPO staining also indicated no changes in neutrophil recruitment (Figure 3D). However, when HFD feeding was prolonged for 6 months, a small yet significant reduction of ALT and AST levels was found in the Sele KO cohort compared with WT mice (Figure 3E). This reduction in liver enzyme levels was in line with a decrease in liver weight and liver to body weight ratio (Figure 3F), suggesting a beneficial effect of knocking out E-selectin in steatosis-associated liver injury. Furthermore, serum glucose levels in HFD-fed Sele KO mice were significantly higher than those in WT animals (Figure 3G), which prompted the question of whether HFD-induced glucose metabolism could be different between Sele KO and WT mice. A glucose tolerance test (GTT) showed similar levels of insulin resistance in HFD-fed WT and Sele KO mice, compared with chow-fed WT mice (Figure 3H). However, the insulin tolerance test (ITT) showed that Sele KO mice had a higher insulin sensitivity than their WT counterparts, which might explain the slight reduction of body weight observed in Sele KO mice. It should be noted, however, that the small differences in liver enzyme levels, body weight, liver weight to body weight ratio, and glucose metabolism were only statistically significant when large animal cohorts (WT, n = 26; Sele KO, n = 31) were compared.

**Deletion of the Sele Gene Reduces Liver Injury and Fibrosis in HFD-Induced NASH by Decreasing Reactive Oxygen Species, p38, and c-Jun-N-Terminal Kinase Activation**

Hepatocyte apoptosis represents one of the major pathogenic events responsible for the transition of steatosis to NASH and was more significant in the HFD-Induced NASH model than in the HFD-induced steatosis model. Therefore, we investigated the effect of Sele KO on liver damage in the HFD-Induced model. Under
these conditions, Sele KO mice had significantly lower serum levels of ALT and AST than WT mice (Figure 4A), without differences in body weight or liver to body weight ratio (Figure 4B). This coincided with a reduction of MPO+ and F4/80+ cells in the liver (Figure 4C–E). Immunocytochemistry staining for ionized calcium binding adaptor molecule 1, which is a pan-macrophage marker, and C-type lectin domain family 4 member F, which marks only tissue-resident Kupffer cells, showed that the ratio between resident Kupffer cells and infiltrated macrophages is the same between WT and Sele KO mice (Figure 4F) although the total number of macrophages in the liver was reduced in Sele KO mice than in WT mice (Figure 4E). This suggests that the observed reduction in hepatic macrophages in Sele KO mice is owing to both a reduction in resident Kupffer cells and infiltrated macrophages. Furthermore, the reduction in hepatic neutrophils and macrophages was not caused by a disturbance in circulating cells because the number of white blood cells (neutrophils, monocytes, leukocytes, and lymphocytes) remained the same in both WT and Sele KO mice (Figure 4F) although the total number of macrophages in the liver was reduced in Sele KO mice than in WT mice (Figure 4E). The observed decrease in death receptor 5 (Dr5), Ly6g, and Adgre1 mRNA levels in Sele KO mice further supported the observed decrease in apoptosis and inflammatory cell infiltration (Figure 5C). In addition, hepatic levels of lipid peroxide 4-hydroxynonenal were lower in Sele KO mice than those in WT mice (Figure 5D and E), suggesting that hepatic oxidative stress is reduced in Sele KO mice. In agreement with these findings, hepatic activation of stress kinases such as p38 and c-Jun-N-terminal kinase (JNK) was lower in Sele KO mice when compared with WT mice, although the levels of apoptosis signal-regulating kinase 1 were comparable between Sele KO and WT controls (Figure 5E).

To further investigate whether E-selectin KO has persistent effects on the progression of NASH, we investigated the alterations in fibrosis and steatosis between Sele KO and WT mice treated with HFD+Cxcl1. Compared with WT mice, Sele KO mice had a strong reduction in Sirius Red staining of collagen fibers (Figure 6A), reduction in mRNA levels of fibrogenic genes, transforming growth factor β (Tgfb) and Acta2 (Figure 6B), and a reduction of α-smooth muscle actin protein (Figure 6C). Determination of liver fat in WT and Sele KO mice under HFD+Cxcl1 conditions did not show any difference between these 2 groups as illustrated by Oil Red O lipid staining (Figure 6D) and by quantitative determination of hepatic triglycerides (Figure 6E). Furthermore, RT-qPCR analysis showed that the expression of fatty acid translocase (Cd36), which
Figure 3. Deletion of the Sele gene slightly reduces liver damage in HFD-fed mice. (A–D) WT (n = 7) and Sele KO (n = 6) mice were subjected to 3-month HFD feeding (3M HFD). (E and F) WT (n = 20–23) and Sele KO (n = 23) mice were subjected to 6-month HFD feeding (6M HFD) or chow diet (n = 13) (WT Chow). Sera and liver tissues were collected and analyzed. 
(A) Serum ALT and AST levels of 3M HFD. (B) H&E staining of liver tissue. (C) Body weight (BW) and liver weight to body weight ratio (LW/BW). (D) MPO immunostaining of liver tissues. (E) Serum ALT and AST levels. (F) BW and LW/BW. (G) Serum glucose levels and (H) GTT and ITT. *P < .05.
Figure 4. Deletion of the Sele gene ameliorates hepatocellular injury in HFD $^{+}$AdCxcl1-induced NASH. WT (n = 7) and Sele KO (n = 6–7) mice were subjected to 3-month HFD feeding plus AdCxcl1 to induce NASH. Blood, sera, and liver tissues were collected and analyzed. (A) Serum ALT and AST levels. (B) Body weight (BW) and liver to body weight ratio (LW/BW). (C–E) MPO and F4/80 immunostaining of liver tissues. (F) Ionized calcium binding adaptor molecule 1 (IBA1) (all macrophages) and C-type lectin domain family 4 member F (CLEC4F) (resident Kupffer cells) immunofluorescence staining and cell ratio determination. (G) Blood leukocyte determination. *P < .05. DAPI, 4',6-diamidino-2-phenylindole.
Figure 5. Deletion of the Sele gene decreases apoptosis, reactive oxygen species, p38, and c-Jun-N-terminal kinase (JNK) stress kinases in HFD-AdCxcl1-induced NASH. WT (n = 7) and Sele KO (n = 6–7) were subjected to 3-month HFD feeding plus AdCxcl1 to induce NASH. Sera and liver tissues were collected and analyzed. (A) Terminal deoxynucleotidyl transferase–mediated deoxyuridine triphosphate nick-end labeling (TUNEL) staining of liver tissues. (B) Western blot determination of poly (adenosine diphosphate-ribose) polymerase (PARP) in liver tissues. (C) RT-qPCR of liver tissues. (D) 4-hydroxynonenal (4-HNE) staining of liver tissues. (E) Western blot analyses of liver tissues and quantification of protein densities. *P < .05.
functions as the main importer of fatty acids into hepatocytes as well as de novo lipogenesis genes, did not differ between WT and Sele KO mice (Figure 6F).

We also tested the role of E-selectin in another model of NASH induced by feeding a Western diet for a 10-week period. The Western diet induced a typical steatotic phenotype and great liver injury in both WT and Sele KO mice (Figure 7A and B). Surprisingly, WT and Sele KO mice did not show any differences in ALT, AST, or liver MPO⁺ cells (Figure 7C and D). Although the Western diet (high-fat, high-cholesterol, and high-sucrose diet) often is suggested as a NASH-inducing diet, liver injury in this model may be caused mainly by high-cholesterol-induced hepatotoxicity and not by neutrophils, which may be why Western diet-induced liver injury was not reduced in Sele KO mice.

Deletion of the Sele Gene Lowers Inflammation, Lipolysis, and Adipokine Secretion in Adipose Tissue in HFD⁺AdCxcl1-induced NASH

Adipose tissue inflammation generally has been associated with the severity of NAFLD. We previously showed that adipocyte death contributes to liver injury by promoting the release of FFA into circulation and inducing hepatic macrophage infiltration and activation. However, how immune cell infiltration in adipose tissue affects the progression of liver injury has not yet been documented. To understand this question, we evaluated the immune cell infiltration in adipose tissue of WT and Sele KO mice after HFD⁺AdCxcl1 challenge. MPO and F4/80 immunostaining (Figure 8A) and RT-qPCR analyses of their corresponding genes (Ly6g and Adgre1) (Figure 8B)
showed that compared with WT mice, Sele KO mice had a reduction of neutrophils and macrophages in epididymal adipose tissue, which correlated with a lower gene expression of E-selectin ligands Cd44 and Selplg and of proinflammatory cytokines Il1b, Ccl2, and Tnfa (Figure 8B). Interestingly, the expression of lipolysis-related gene Hsl and Atgl also was reduced markedly in Sele KO mice (Figure 8C), which correlated with a reduction of serum FFA (Figure 8D).

There is increasing evidence that alterations in proinflammatory adipokines produced by adipose tissue play a role in adipose tissue inflammation and contribute to the development of NASH. Therefore, we investigated the expression patterns of multiple adipokines in adipose tissue and found that the expression of Retn, insulin-like growth factor-binding protein 1 (Igfbp1), leptin (Lep), retinol binding protein 4 (Rbp4), and S100 calcium-binding protein A8 (S100a8) and S100a9 mRNA levels was reduced in Sele

Figure 7. E-selectin KO does not reduce liver injury in Western diet (WD)-fed mice. WT (n = 6) and Sele KO (n = 6) mice were put on a WD for 10 weeks to induce NASH. Serum and liver samples were collected and analyzed. (A) H&E staining of liver tissues. (B) Body weight (BW) and liver weight to body weight (LW/BW) ratio. (C) Serum ALT and AST levels. (D) MPO immunostaining of liver tissues.
Figure 8. Genetic deletion of the Sele gene reduces inflammation, lipolysis, and proinflammatory adipokine secretion in adipose tissue from HFD + AdCxc1f-induced NASH. WT (n = 4–7) and Sele KO (n = 4–7) were subjected to 3-month HFD feeding plus AdCxc1f to induce NASH. Sera and epididymal adipose tissues were collected and analyzed. (A) MPO and F4/80 immunostaining of adipose tissues. (B, C, and E) RT-qPCR of adipose tissues. (D) Determination of serum FFAs. (F) Determination of serum proinflammatory adipokines. *P < .05.
KO mice compared with WT controls under the HFD\textsuperscript{+AdCxcl1} challenge (Figure 8E). In addition, we investigated the serum protein levels of multiple adipokines and found that several proteins, including the S100A8/A9 dimer, multiple members of the IGFBP family, resistin, angiotensinogen, insulin, and RBP4, were present at lower levels in KO mice compared with WT mice (Figure 8F).

**E-Selectin-Dependent S100A8/A9 Expression Is Up-regulated in Adipose Tissue During NASH**

The earlier-described data suggest that reduction of neutrophil and macrophage recruitment in adipose tissue via deletion of Sele lessens the NASH phenotype by reducing tissue inflammation and lipolysis. To understand the mechanism by which neutrophils in adipose tissue exacerbate steatosis-to-NASH progression, we focused on neutrophil-enriched proteins S100A8 and S100A9, which were reduced markedly in adipose tissue and serum from Sele KO mice compared with WT mice as described earlier. We first analyzed transcriptomic data sets of adipose tissue\textsuperscript{1} from patients and found a significant up-regulation of S100A8 and S100A9 in the adipose tissue samples of NASH patients, compared with tissue of patients with normal livers (Figure 9A). Inversely, the expression of these genes in the liver was down-regulated in NASH patients compared with patients with steatosis or normal liver (Figure 9B) based on analysis of transcriptomic data sets of NASH liver samples.\textsuperscript{25}

The role of S100A8/A9 was investigated further using the HFD\textsuperscript{+AdCxcl1}-induced NASH model. Western blot analysis and histochemical staining of adipose tissue of mice under HFD\textsuperscript{+AdCxcl1} conditions showed much higher S100A8/A9 levels compared with HFD-fed mice (Figure 9C), supporting the clinical data in Figure 9A. However, this induction was much less evident in the liver (Figure 9D). Immunohistochemistry analyses showed that strong S100A8/A9 protein staining was observed in adipose tissue of WT mice under HFD\textsuperscript{+AdCxcl1} conditions and such staining was diminished markedly in Sele KO mice (Figure 9E and F). Surprisingly, the liver tissues from HFD\textsuperscript{+AdCxcl1}-treated WT mice only had weak S100A8/A9 protein staining, which was slightly reduced in Sele KO mice (Figure 9E). Finally, Western blot analyses also showed strong expression of S100A8/A9 protein in adipose tissue but weak expression in liver tissues, with reduced levels in Sele KO mice compared with WT mice (Figure 9G).

**S100A9 Inhibitor Ameliorates NASH in Mice via Inhibition of Lipolysis in Adipose Tissue**

To examine the roles of S100A8/A9 in neutrophil-driven NASH, we treated HFD\textsuperscript{+AdCxcl1}-challenged mice with the S100A9 inhibitor PAQ (schematic in Figure 10A). Administration of 10 or 20 mg/kg PAQ markedly reduced serum ALT and AST levels (Figure 10B) without affecting body weight or liver to body weight ratio (data not shown). This reduction in liver injury correlated with a decreasing trend in mRNA levels of the apoptotic marker Dr5 and a significant reduction of inflammatory markers Il1b, Ccl2, Ifng, and Tnfa. Moreover, Agrp1 and Lypg mRNA levels, which encode macrophage F4/80 and neutrophil lymphocyte antigen 6 complex locus G6D marker proteins, respectively, remained unchanged (Figure 10C), indicating that the amount of these cells in the liver was not affected by the treatment. In addition, hepatic expression levels of fibrogenic genes including Acta2, Col1a1, Col1a2, Col3a1, and Col4a1 also were ameliorated when the highest concentration of PAQ was administered (Figure 10D). In adipose tissue, expression of the apoptotic and inflammatory markers was reduced after administration of PAQ, but this reduction did not reach statistical significance (Figure 10E). Interestingly, the expression of lipolysis-related genes including Hsl, Atg1, and Plin1 in adipose tissue was reduced markedly in the 20 mg/kg PAQ group (Figure 10F). Such reduction in lipolysis-related genes correlated with a significant decrease in serum FFA (Figure 10C). Furthermore, a trend in reduction of adipokine mRNA levels also was observed, although these differences were not statistically significant (Figure 10F). However, treatment with 10 mg/kg PAQ significantly reduced the serum levels of resistin (Figure 10H).

To further pinpoint the effect of S100A8/A9 in adipose tissue, we exposed differentiated adipocytes to a recombinant mouse S100A8/A9 dimer in vitro and found that such treatment markedly up-regulated lipolysis-related gene expression, as indicated by a significant increase of Hsl and Atg1 (Figure 10I). Exposure to S100A8/A9 also significantly up-regulated Retn and showed an increasing trend in Lep and Rbp4 expression in adipocytes in vitro.

**Discussion**

The risk for adverse outcomes in patients with simple steatosis is low, but can increase rapidly when this condition evolves to NASH.\textsuperscript{26} The exact mechanisms that drive this steatosis-to-NASH progression are still not fully elucidated, however, triggers of liver inflammation can have their origins in the liver itself or in another organ such as adipose tissue or the gut.\textsuperscript{27} We have shown previously that over-expression of the neutrophil chemokine CXCL1 causes neutrophil infiltration and NASH in HFD-fed mice by producing reactive oxygen species and activating the stress kinase p38,\textsuperscript{28,29} However, exactly how neutrophils in adipose tissue affect the development and progression of NASH has not been documented.

In the current study we uncovered that the expression of E-selectin, which plays a crucial role in the initial stages of neutrophil recruitment,\textsuperscript{10} is increased during NASH at markedly higher levels in adipose tissue than in the liver, suggesting that E-selectin–dependent inflammation occurs predominantly in adipose tissue. Our data further showed that several inflammatory cytokines and FFAs were able to up-regulate E-selectin in endothelial cells. It is known that adipose tissue likely has higher levels of FFAs than liver tissue, which probably contributes, at least in part, to the greater induction of E-selectin in adipose tissue when compared with liver tissue.

E-selectin was documented previously to be implicated in inflammation of the liver\textsuperscript{11,26} and adipose tissue.\textsuperscript{40}
Increased soluble E-selectin in plasma has been shown to be associated with NAFLD severity in patients. However, how E-selectin contributes to the NAFLD pathogenesis remains unknown. Now, we provide evidence supporting that this molecule indeed plays a specific regulating function during the steatosis-to-NASH transition. First, E-selectin
induction in adipose tissue or the liver was observed during HFD+AdCxcl1-induced NASH but not simple steatosis, hinting at a NASH-specific function of this adhesion molecule. Second, genetic deletion of the Sele gene resulted in a significant decrease in liver injury in HFD+AdCxcl1-induced NASH, as indicated by a decrease in serum aminotransferase levels and liver apoptosis, which corroborated with reduced activation of p38 and JNK stress kinases. Third, decreased liver fibrosis in Sele KO mice further substantiated a reduction in NASH phenotype associated with E-selectin loss of function. Interestingly, the effect of E-selectin ablation overrides the effect of CXCL1 in inducing neutrophil infiltration in the liver. We speculate that the chemoattractant action of CXCL1 induces the transport of neutrophils to the liver, but that the adhesion of these cells to sinusoidal endothelial cells is compromised in the Sele KO mice and, as a result, the cells that remain in circulation are removed by the blood flow.

E-selectin KO also showed a reduction in serum aminotransferase levels on a HFD-induced steatosis mouse model. However, this reduction was observed only when the period of HFD was prolonged (6 months instead of 3 months), and was only statistically significant when a high number of mice in each group was used. Because prolonged HFD feeding has been associated with an increase of inflammation and progression of NAFLD, it is reasonable to assume that these 6-month HFD-fed mice already have a more advanced form of NAFLD in which more inflammation is present and therefore the role of E-selectin becomes more prominent.

As per the leukocyte adhesion cascade paradigm, a coordinated sequential action of several adhesion molecules is required for the recruitment of circulating leukocytes to areas of inflammation. Therefore, besides E-selectin, other adhesion molecules also may play a role in steatosis-to-NASH progression. It recently was documented that hepatic mRNA levels of L-selectin, which is expressed on most types of lymphocytes, but particularly on neutrophils, was induced in patients with steatosis and increased further in patients with NASH. The same study also reported that deletion or therapeutic blockade of L-selectin prevented the development and progression of steatohepatitis in HFD- and MCD-diet-fed mice. In our study, we found that the modulation of SELL expression in patients with NASH was markedly lower than that of SEL, which also was more specific to the NASH condition because no induction was observed in steatosis samples compared with normal livers. Thus, we surmise that E-selectin plays a more specific role than L-selectin in steatosis-to-NASH progression.

Similar to the liver, a clear reduction of inflammation also was observed in adipose tissue of Sele KO mice during HFD+AdCxcl1-induced NASH. Compared with WT mice, Sele KO mice had a decrease in infiltration of neutrophils and macrophages, and a reduction in the expression and secretion of proinflammatory adipokines such as IGFBP, resistin, leptin, RBP4, and ANGPTL3 in adipose tissue. These changes probably contribute to the improved NASH phenotypes in Sele KO mice because increased serum values of resistin, leptin, RBP4, and ANGPTL3 have been suggested to promote NASH pathogenesis, and an increase of IGFBPs was associated with advanced fibrosis. Moreover, we found that S100A8/A9 was highly induced in adipose tissue of mice under NASH conditions and markedly reduced if Sele KO mice were used. These observations were much less prominent in the liver, substantiating the differences in E-selectin expression between adipose tissue and the liver and the consequential difference in S100A8/A9 levels in these tissues. S100A8 and S100A9 proteins are unstable and therefore most often exist in the form of a stable S100A8/A9 heterodimer, which comprises approximately 45% of the cytoplasmic proteins in neutrophils. S100A8/A9 has been documented to stimulate leukocyte recruitment and induce cytokine secretion. In the current study, we showed that S100A8/9 plays an important role in promoting NASH as treatment with the S100A8/9 inhibitor PAQ considerably improved the NASH phenotype.

Another important observation from the current study was the striking reduction of lipolysis-related genes in adipose tissue of Sele KO mice during HFD+AdCxcl1-induced NASH, which resulted in a decrease in secretion of FFA and proinflammatory adipokines that are known to promote liver injury. Increased FFA levels in the blood are known to directly induce hepatotoxicity and contribute to NASH pathogenesis. Therefore, our data suggest that E-selectin plays an important role in inducing lipolysis in adipose tissue and increasing circulating FFAs during NASH, contributing to NASH pathogenesis. The role of E-selectin in inducing lipolysis probably is mediated via the neutrophil recruitment and subsequent production of S100A8/A9 because inhibition of S100A8/A9 down-regulated lipolysis-related gene expression in adipose tissue, while treatment with S100A8/A9 protein up-regulated expression of these genes in cultured adipocytes.

Altogether, our data indicate that E-selectin-dependent neutrophil recruitment in both the liver and adipose tissue is an important driver of steatosis-to-NASH progression (Figure 10f). E-selectin, however, triggers distinct pathogenic processes in the liver and in adipose tissue that

**Figure 9. (See previous page).** S100A8/A9 is up-regulated in adipose tissue but not in the liver during NASH, which is dependent on E-selectin. (A and B) Normalized gene expression (microarray data sets obtained from Du Plessis et al.[17] and Lake et al.[18] in visceral adipose tissue (AT) and liver of patients suffering from steatosis (nAT = 7, nL = 10) and NASH (nAT = 13, nL = 16) compared with healthy controls (normal) (nL = 19, nAT = 8). (C and D) WT mice were subjected to 3-month HFD plus AdCxcl1 (HFD+AdCxcl1) to induce NASH or HFD+AdGfp (HFD) as controls. Adipose and liver tissues were collected and analyzed. Western blot analyses of S100A8/A9 dimer in adipose and liver tissues. (E–G) WT and Sele KO were subjected to 3-month HFD plus AdCxcl1 to induce NASH. Adipose and liver tissues were collected and analyzed. (E, left; and F) Immunostaining analyses of S100A8/A9 in adipose tissue and (E, right) liver tissues. (G) Western blot analyses of S100A8/A9 dimer of adipose tissues (top panel) and liver tissues (bottom panel). *P < .05.
together exacerbate the steatosis-to-NASH progression. Although it is difficult to weigh the detrimental effect of E-selectin in each organ, we speculate that it plays a more prominent role in adipose tissue because E-selectin expression is increased during NASH at markedly higher levels in adipose tissue compared with the liver. Targeting E-selectin-induced adipose tissue inflammation therefore may represent a potential novel therapy for treatment of NASH.
Material and Methods

Animal Experiments

All animal experiments were approved by the National Institute on Alcohol Abuse and Alcoholism’s Animal Care and Use Committee and conducted in accordance with National Institutes of Health guidelines. C57BL/6J and B6.129S4-Sele tm1Dmii /J (Sele KO) mice originally were obtained from The Jackson Laboratory (Bar Harbor, ME) and further bred at the National Institute on Alcohol Abuse and Alcoholism animal facility.

HFDΔCxcl1-Induced Mouse Model of NASH

To generate CXCL1-induced NASH, 10- to 12-week-old male mice were fed an HFD (60 kcal% fat, D12492; Research Diets, New Brunswick, NJ) for 3 or 4 months and injected via tail vein with adenovirus-Cxcl1 (Applied Biological Materials, Richmond, Canada). The mice then were kept on a HFD for 2 additional weeks after AdCxcl1 injection before killing for analysis. The control mice were fed an HFD and received a tail vein injection of AdGfp instead of AdCxcl1. Paquinimod (Novus Biologicals, Centennial, CO) treatment was administered at 2 different doses, namely 10 and 20 μg/kg, by intraperitoneal injection every second day starting 2 days after AdCxcl1 injection. The mice were kept on a HFD during the treatment period and were killed 2 weeks after AdCxcl1 infection.

Western Diet

To generate diet-induced steatohepatitis, 10- to 12-week-old C57BL/6J male mice were put on an Amylin Liver NASH D09100301 diet (40 kcal% fat, 20 kcal% fructose, 2% cholesterol; Research Diets), mimicking a Western diet, for 10 weeks. Mice fed a chow diet, for 10 weeks after AdCxcl1 injection.

Serum ALT and AST

Serum ALT and AST levels were determined using the Catalyst Dx Chemistry Analyzer (IDEXX Laboratories, Westbrook, ME) or ALT Kinetic and AST Kinetic (Teco Diagnostics, Anaheim, CA) according to the manufacturer’s instructions.

Serum FFAs

Serum FFAs levels were determined using the Free Fatty Acid Quantification Colorimetric/Fluorometric Kit (Biovision, Milpitas, CA) according to the manufacturer’s instructions.

Serum Glucose, GTT, and ITT

Serum glucose was measured using a glucometer (Bayer Corporation, St. Louis, MO). For the GTT and the ITT, mice were fasted for 16 hours or 4 hours, respectively, and allowed free access to water. Glucose (2 g/kg; Sigma Aldrich, St. Louis, MO) and insulin (1 U/kg, Humalog; Lilly, Indianapolis, IN) were injected intraperitoneally, respectively. Tail blood glucose levels were measured at 0, 15, 30, 45, 60, 90, and 120 minutes after glucose or insulin injection.

Serum Cytokines, Chemokines, and Adipokines

Serum levels of mouse CXCL1 were determined using the Mouse CXCL1/KC Quantikine enzyme-linked immunosorbent assay (ELISA) kit and serum levels of mouse S100A8/A9 were quantified using the Mouse S100A8/S100A9 Heterodimer DuoSet ELISA (both from R&D Systems, Minneapolis, MN) following the manufacturer’s instructions. Resistin serum levels of were measured using the RayBio Mouse Resistin ELISA Kit (RayBiotech, Peachtree Corners, GA). The Proteome Profiler Mouse Adipokine Array Kit (R&D Systems) was used for the determination of IGFBPs, resistin, ANGPTL3, dipeptidyl peptidase 4, leptin, and RBP4 in serum samples pooled per group. The assay was repeated to generate a total of 4 measurements per group.

Blood Leukocyte Determination

A complete blood count test for blood leukocyte determination was performed on anticoagulated blood using a Hemavet 950 FS Hematology Analyzer (Drew Scientific, Dallas, TX).

Liver Triglyceride Determination

Approximately 50 mg of the frozen liver tissues were used for determination of triglyceride content with the Triglyceride Colorimetric Assay Kit (Cayman Chemical, Ann Arbor, MI), according to the manufacturer’s instructions.

Immunohistochemistry and Immunofluorescence Staining

Mouse epididymal adipose tissue and liver samples were collected and fixed in formalin, unless otherwise specified. Paraffin sections (4-μm thick) were cut and deparaffinized for histologic analysis. H&E staining (Richard-Allan Scientific, Kalamazoo, MI), Sirius Red staining (Sigma), and terminal deoxynucleotidyl transferase–mediated deoxyuridine triphosphate nick-end labeling staining (ApopTag Peroxi-dase In Situ Apoptosis Detection Kit; Millipore, Burlington, MA) were performed according to the manufacturers’ instructions.

Figure 10. (See previous page). Inhibition of S100A9 ameliorates liver damage and inflammation in NASH by lowering lipolysis and adipokines in adipose tissue. WT mice were subjected to 4-month HFD feeding, followed by injection of AdCxcl1 and treatment with 2 different doses of PAQ: 10 μg/kg (PAQ10) (n = 7) or 20 μg/kg (PAQ20) (n = 6), or vehicle control (VC) (n = 5). Serum, liver, and adipose tissues were collected and analyzed. (A) Schematics for the administration of PAQ to HFDΔCxcl1-challenged mice. (B) Serum ALT and AST levels. (C and D) RT-qPCR of liver tissues. (E and F) RT-qPCR of adipose tissues. (G and H) Determination of serum FFAs and serum resistin. (I) RT-qPCR analyses of differentiated pre-adipocytes exposed to vehicle control or S100A8/A9. (J) Schematic illustration of the proposed role of E-selectin and S100A8/A9 in the liver and adipose tissue during neutrophil-induced NASH. *P < .05.
instructions. For immunohistochemistry, heat-induced epitope retrieval was performed in 3% citrate buffer followed by incubation of the paraffin-embedded sections in 3% H2O2. Blocking of nonspecific binding was performed using 3% normal goat serum for 10–60 minutes. The sections then were incubated with primary antibodies (Table 1) overnight at 4°C and subsequently with anti-mouse or anti-rabbit secondary antibodies (SignalStain Boost Immunohistochemistry Detection Reagent; Cell Signaling Technology, Danvers, MA) for 1 hour at room temperature. The staining was visualized using the Vectastain Elite ABC Staining Kit and 3,3′-diaminobenzidine tetrahydrochloride Peroxidase Substrate Kit (Vector Laboratories, Burlingame, CA) according to the manufacturers’ instructions. Nuclear staining then was performed using hematoxylin (Richard-Allan Scientific), upon which the slides were dehydrated and mounted with Fisher Chemical Permount Mounting (Thermo Fisher Scientific, Waltham, MA). For immunofluorescence staining, after primary antibody incubation overnight, samples were incubated with secondary antibodies (goat anti-rabbit IgG, Alexa Fluor 488; streptavidin, Alexa Fluor 555 conjugate; Thermo Fisher Scientific) for 1 hour at room temperature. Nuclear staining was obtained by incubation with 1 mg/mL 4′,6-diamidino-2-phenylindole for 5 minutes. Images were acquired using either a BX41 microscope for bright field images (Olympus, Bethlehem, PA) or a LSM 710 confocal microscope (Zeiss, Thornwood, NY) for fluorescent images.

**Western Blot**

Liver and adipose tissues were homogenized in RIPA buffer containing a cocktail of protease inhibitors (Santa Cruz Biotechnology, Dallas, TX) according to the manufacturer’s instructions. Protein extracts were quantified using the Pierce BCA Protein Assay Kit (Thermo Fisher) and adjusted with RIPA before loading onto 4%-12% Bis-Tris protein gels (Bio-Rad, Hercules, CA) and transferred to nitrocellulose membranes (Thermo Fisher). Primary antibodies used in the Western blots can be found in Table 1. Secondary horseradish-peroxidase–conjugated antibodies (Santa Cruz Biotechnology) were used. Protein bands were visualized with SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Fisher).

**Oil Red O Staining**

Liver samples were snap-frozen in liquid nitrogen after killing and embedded in Tissue-Plus OCT Compound (Thermo Fisher Scientific) for cryosectioning. Cryosections (5-μm thick) were washed with 60% isopropanol for 1 minute and stained with 0.6% Oil Red O solution (Sigma-Aldrich) for 30 minutes. After washing with 60% isopropanol and distilled water (3 times each), the nuclei were counterstained with hematoxylin and the slides were mounted for microscopy.

**RT-qPCR**

Total RNA was purified from snap-frozen adipose tissue, liver tissues, or cell cultures using TRIzol Reagent (Thermo Fisher) and Qiagen RNEasy Mini kit protocol (Qiagen, Valencia, CA) according to the manufacturer’s instructions. Reverse transcription of RNA into complementary DNA was performed using the High-Capacity Complementary DNA Reverse Transcription kit (Thermo Scientific, Waltham, MA) and cDNA was amplified using the SmartCycler system and Power SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA). The primers used are listed in Table 2.

**Table 1. Antibodies**

| Primary antibody | Dilution       | Source                      |
|-----------------|---------------|-----------------------------|
| 4-HNE (mouse)   | 1:400 (staining) 1:1000 (WB) | Genox, Baltimore, MD        |
| ASK1 (rabbit)   | 1:1000        | Thermo Fisher               |
| p-ASK1 (Thr838) (rabbit) | 1:5000       | Thermo Fisher               |
| CD31 (rabbit)   | 1:200         | Cell Signaling Technologies |
| CLEC4F (rat)    | 1:100         | R&D Systems                 |
| E-selectin (biotinylated) | 1:200 (staining) 1:5000 (WB) | R&D Systems               |
| F4/80 (rabbit)  | 1:400         | Cell Signaling Technology   |
| IBA1 (rabbit)   | 1:1000        | Avantor/VWR, Philadelphia, PA |
| JNK (rabbit)    | 1:1000        | Cell Signaling Technology   |
| p-JNK (Thr183/Tyr185) (rabbit) | 1:1000       | Cell Signaling Technology   |
| MPO (rabbit)    | 1:1           | Biocare Medical, Concord, CA |
| p38 (rabbit)    | 1:1000        | Cell Signaling Technology   |
| p-p38 (Thr180/Tyr182) (rabbit) | 1:1000    | Cell Signaling Technology   |
| S100A8/A9 (mouse) | 1:500 (staining) 1:5000 (WB) | Novus Biologicals           |
| α-SMA (mouse)   | 1:5000        | Sigma-Aldrich               |
| β-actin (mouse) | 1:5000        | Abcam, Cambridge, MA        |

4-HNE, 4-hydroxynonenal; ASK1, apoptosis signal-regulating kinase 1; α-SMA, α-smooth muscle actin; CLEC4F, C-type lectin domain family 4 member F; IBA1, ionized calcium binding adaptor molecule 1; JNK, c-Jun-N-terminal kinase; p-ASK1, phosphorylated ASK1; p-JNK, phosphorylated JNK; p-p38, phosphorylated p38 kinase; WB, Western blot.
Real-time PCR was performed using an ABI7500 RT-PCR system (Applied Biosystems, Foster City, CA). 18s, Gapdh, Actb, and Apob were used as normalizing housekeeping genes. All primers used for RT-qPCR are listed in Table 2.

Isolation and Culture of Mouse Primary Hepatocytes
Mice were anesthetized with 30 mg/kg pentobarbital sodium intraperitoneally. The portal vein was cannulated, and the liver was perfused with ethylene glycol tetra-acetic acid and subsequently with 0.075% collagenase (Worthington, Lakewood, NJ) solution. The obtained mixture then was incubated on a shaker at 100 rpm for 10 minutes at 37°C. After the digestion the cell suspension was filtered through a 70-μm nylon mesh and primary hepatocytes were collected after centrifugation at 500/C2 g for 5 minutes. Hepatocytes were plated on cell culture recipients coated with Rat Tail Collagen Type I (Corning, Corning, NY) at a density of ±50,000 cells/cm² and further cultures in cell culture medium composed of Dulbecco’s modified Eagle medium, 10% fetal bovine serum, and penicillin-streptomycin (all from Thermo Fisher Scientific). After 4 hours, culture media were replaced to remove nonadhered cells and debris. The cells then were exposed to palmitic acid (600 μmol/L) (Sigma) or vehicle control for 24 hours. Hereafter, the cell culture medium was removed, and the cells were cultured for a further 24 hours with fresh culture medium, which then was reconditioned to be used in TSEC experiments.

Isolation of Neutrophils
Bone marrow of C57BL/6-Tg(CAG-EGFP)1Osb/J mice (The Jackson Laboratory), which have widespread enhanced green fluorescent protein (eGFP) fluorescence, with the exception of erythrocytes and hair, was collected from femur and tibia in phosphate-buffered saline (Thermo Fisher Scientific). After filtration through a 70-μm cell strainer the cell suspension was centrifuged for 5 minutes at 300/C2 g and the obtained cell pellet was incubated with ACK lysing buffer (BioWhittaker, Walkersville, MD) for 1 minute to

| Table 2. Primers |
|-----------------|-----------------|-----------------|
| **Gene**       | **Forward, 5’-3’** | **Reverse, 5’-3’** |
|----------------|-----------------|-----------------|
| Acc1           | TGGACAGACTGATCCGAGAAAG | TGGAGAGCCCAACACACA |
| Acta2          | TCTCTGCCGAGTTTATCCGATA | GGGTCCAGCTTCTTCATCAGTC |
| Adgre5         | ATCCTACCATCTTACGAGCC | GGGCAGCAGTCGACCAGATAG |
| Atg3           | CCACTCCGGCGCCATGCCAGG | TAATGTTGCGGCTTCTTC |
| Ccl2           | TCTGAGGCCATCCTTCTTGG | TCAGCCAGATCAGTTCAGGC |
| Cd36           | CCTGCAACATGCAAGGAGA | GCGACATGATGAAAGCAG |
| Cd44           | TGAAATCGAGTAGTGGGT | GGTAGAGGCTTGAGAACAT |
| Dlat1          | GACGGCTACTGGGATCAG | TCACACACACACATCAAAG |
| Dr5            | GGGGCTTGCGTGTTGTCACT | GGGGCTTGCGTGTTGTCACT |
| Fasn           | GGAGGGAGTGATAGGCGGTAT | TGGGTAATCCATAGGCCAG |
| F4/80          | TTTGCCATGCGGTCCAGTC | GCAAGGAGGCAGAGGATAGGT |
| Gapdh          | AGCCGCCGCCATTTGCAGG | GGGCCCTTGCCCGTGAGT |
| Hsl            | CCTGAAAGATGCTGAGC | GGAGAGTCTGGGAGAACAG |
| Icam1          | CAATTTCTCATGCCGACAG | AGCTTGAGAATGAGAACAG |
| Igf2bp1        | ATCAGCCACTTGTGGAGAC | TGGAGCACTAATCTATCGG |
| Il1b           | TGACAGAGGCTGACAGAACAA | CATCGAGGCCAGGAGGAAAC |
| Il6            | ACAATCGGAGAGTGCTAATACCAT | TTGCCATTGGCAACACTTTC |
| Lep            | GAGACCCTCGGCTGCTGCTTGT | CGGTGCTGTTGAAATGCTTTG |
| Ly6e           | TCGGGGTCGAGATGAGA | CAGAGATGCTGGGGAGATG |
| Rbp4           | AGTACAGGCTGCGACAG | GAGAAGAGTGCTGAGGAGATG |
| Retn           | AAGAACCTTTCTGACCTTTCTCT | GTCCAGCAATTAAAGGGAGG |
| S100a8         | AAATCGGCTCCCTCATAAG | CCCACTTTTATCACCAGC |
| S100a9         | ATACTCGGAGAAAGGAGCGCC | TCAGATGCTTCTGTGAGGG |
| Scd1           | TCTCTTGCGATACCTCTGTGCT | CGGTGAGAATGGTGGCTT |
| Sele           | CCAATCTGAAATCATCCAGG | GAGTTCTGGGTGGTGAGT |
| Sell           | TACAGCTTGGGGTTTCTCTT | CATCTTGAGGAGGAGGAGT |
| Selp           | GCCAGGTGATGCTGGGATGAA | GGGCGAGATGCTGGGAGA |
| Tgfb            | CAGGCGGCTGGTATCCTA | GAGAACGCTGAGGAGAAG |
| Traf3          | CTCCTCGACATCATTTTTCTT | GCTAGAGGCTGGGTAGTG |
| Vcam1          | TGAACCCAAACAGAGGCAG | GGATCTCCCTGACCTCAGG |
remove red blood cells. The neutrophils were isolated by magnetic activated cell sorting using a Neutrophil Isolation Kit (Miltenyi Biotec, San Diego, CA) according to the manufacturer’s instructions.

**TSEC Culture**

TSECs were kindly provided by Dr Vijay Shah (GI Research Unit, Mayo Clinic, Rochester, MN) and cultured as previously described using Endothelial Cell Medium (SkeCell Research Laboratories, Carlsbad, CA). TSECs cultured in 24-well plates were exposed for 24 hours to palmitic acid (600 μmol/L), IL1β (10 ng/mL), tumor necrosis factor α (50 ng/mL), transforming growth factor β (10 ng/mL), CXCL1 (100 ng/mL), or supernatant from primary hepatocytes that had been exposed previously to palmitic acid (600 μmol/L) or vehicle for 24 hours.

**Co-culture of TSECs With Freshly Isolated Neutrophils**

TSECs were seeded into 8-well chamber slides and cultured until 100% cell confluence was reached. The cells then were exposed for 24 hours to inflammatory triggers solubilized in culture medium (palmitic acid, 600 μmol/L; IL1β, 20 ng/mL; and IL6, 100 ng/mL) supernatant from primary hepatocytes that had been previously exposed to palmitic acid (600 μmol/L) or vehicle for 24 hours. After medium refreshment, freshly isolated neutrophils were added (200,000 cells/well) to activated TSECs and further co-cultured for 9 hours. Hereafter, the cell supernatants were aspirated and the cells were thoroughly rinsed with phosphate-buffered saline (Thermo Fisher Scientific) to remove all nonadhered cells. Adhered neutrophils to TSEC monolayers were visualized using an Axio Vert A1 fluorescence microscope (Zeiss).

**Statistical Analysis**

All data were analyzed using GraphPad Prism software (v. 5.0a; GraphPad Software, La Jolla, CA) and are expressed as the means ± SEM. A Student t test was performed to compare values obtained from 2 groups and a 1-way analysis of variance, followed by the Tukey or Fisher post hoc test, was used to compare values obtained from 3 groups. Results with a P value less than .05 were considered significantly different.

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