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High-Fat Diet—Induced Adipose Tissue and Liver Inflammation and Steatosis in Mice Are Reduced by Inhibiting Sialidases

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Darrell Pilling, Tejas R. Karhadkar, and Richard H. Gomer

From the Department of Biology, Texas A&M University, College Station, Texas

High-fat diet (HFD)—induced inflammation and steatosis of adipose tissue and liver are associated with a variety of serious health risks. Sialic acids are found as the distal terminal sugar on glycoproteins, which are removed by sialidases (neuraminidases). In humans and mice, pulmonary fibrosis is associated with up-regulation of sialidases, and injections of sialidase inhibitors attenuate bleomycin-induced pulmonary fibrosis. Sialidase levels are altered in obese rodents and humans. This report shows that for mice on an HFD, injections of the sialidase inhibitor N-acetyl-2,3-dehydro-2-deoxyneuraminic acid inhibit weight gain, reduce steatosis, and decrease adipose tissue and liver inflammation. Compared with control, mice lacking the sialidase neuraminidase 3 have reduced HFD-induced adipose tissue and liver inflammation. These data suggest that sialidases promote adipose and liver inflammation in response to a high-fat diet. *(Am J Pathol 2021, 191: 131—143; https://doi.org/10.1016/j.ajpath.2020.09.011)*

In the United States, approximately 40% of adults are obese (body mass index, ≥ 30 kg/m^2), leading to an estimated annual medical cost of $150 billion and approximately 200,000 deaths.[1—3] Obesity is one of the most prominent risk factors for many chronic diseases, including type 2 diabetes, cardiovascular disease, and nonalcoholic fatty liver disease. Approximately 25% of adults in highly industrialized nations have nonalcoholic fatty liver disease, with approximately 5% of them having nonalcoholic fatty liver disease with liver inflammation (hepatitis), a more serious condition called nonalcoholic steatohepatitis.[4—6] Up to 40% of individuals with nonalcoholic steatohepatitis progress to advanced liver fibrosis and eventually cirrhosis.[7]

Obesity is the accumulation of body fat as the result of excessive food intake and/or lack of exercise. Obesity induces adipocyte metabolic dysregulation and the production of inflammatory cytokines, leading to systemic metabolic dysregulation, such as the inability to effectively control systemic glucose levels (insulin resistance), elevated lipid levels (dyslipidemia), and immune cell recruitment to, and activation in, adipose tissue and liver (inflammation).[8] Excess calories lead to elevated circulating levels of glucose and free fatty acids, which force adipocytes to accumulate more lipid and expand in size, leading to increased oxidative stress in adipocytes and local hypoxia of the tissue, due to the inability of oxygen to diffuse across the tissue.[8,9] These processes lead to adipocyte cell death, initiating the activation of adipose tissue macrophages.[9,10] In the liver, excess calories lead to Kupffer cell (hepatic macrophage) activation, which promotes inflammation and increased hepatocyte fatty acid synthesis, leading to hepatic steatosis (abnormal retention of lipids within the hepatocytes) and eventual fibrosis or cirrhosis.[8,11]

Sialic acids are often found as the distal terminal sugar on the oligosaccharide chains of glycoconjugates, such as glycoproteins. Sialidases (alias neuraminidases) are enzymes that remove this sialic acid from glycoconjugates.[12] Neuraminidase (NEU) 1 to 4 are the four sialidases seen

in mammals.[13] N-acetyl-2,3-dehydro-2-deoxyneuraminic acid (DANA) inhibits mammalian sialidases.[14] Previous studies have found that injections of DANA or lack of NEU3 both attenuate bleomycin-induced lung fibrosis in mice.[15,16] The role of sialidases in the regulation of high-fat diet−induced obesity is unclear, with up-regulation or down-regulation of different sialidase proteins in different tissues, suggesting a complex association between obesity and sialidases.[17−19] Changes in sialidase levels appear to lead to the dysregulation of insulin signaling and glucose metabolism.[18,19]

Because excess calories lead to adipose tissue and liver inflammation, steatosis, and fibrosis, and because injections of DANA and lack of endogenous NEU3 both inhibit bleomycin-induced lung inflammation and fibrosis, this study examined whether injections of DANA and/or endogenous NEU3 could inhibit obesity-induced adipose tissue, liver inflammation, and steatosis in a mouse model.

Materials and Methods

Mouse Model of Obesity

All procedures were done with approval of the Texas A&M University institutional animal care and use committee. The 12- to 16-week−old male C57BL/6 mice (number 000664; Jackson Laboratory, Farmington, CT) were fed standard rodent chow (15% kcal fat; Teklad 8604; Envigo, Madison WI), and obese C57BL/6 mice (number 380050; Jackson Laboratory) were fed a high-fat diet from 6 weeks of age (60% kcal fat; D12492 formula; Research Diets, New Brunswick, NJ). The 12-week−old male C57BL/6-backcrossed $Neu3^{-/-}$ knockout mice ($Neu3^{-/-}$) strain B6.129-NEU3[tm1Yamk] mice[20] were also fed standard and HFD chow. Mice were placed on the specified diets for 6 weeks before the start of treatment. Mice were randomly assigned to dietary and treatment groups by personnel uninvolved with the study. Mice were housed with a 12-hour/12-hour light-dark cycle with free access to food and water. All procedures were performed between 9 AM and noon.

The i.p. injections of phosphate-buffered saline (PBS) or DANA (EMD-Millipore, Burlington, MA) at 10 mg/kg in PBS were given every 48 hours, as described previously.[15] For glucose tolerance tests, mice were fasted for 16 hours before i.p. injections of 1.5 g/kg glucose (Amresco, Solon, OH) in PBS, as described elsewhere.[21] Blood glucose levels were measured before glucose administration (0 minutes), and at 20, 40, 60, 90, and 120 minutes after the injection using commercial blood glucose test strips (CVS Pharmacy, Woonsocket, RI). Mice were euthanized 35 days after initiation of treatment by asphyxiation with CO_2 at 3 L/minute.[22]

Histology and Antibody Staining

Following euthanasia, organs, including epididymal white adipose and interscapular brown adipose tissue, liver, spleen, lungs, and kidneys, were weighed before processing. Pieces of adipose tissue and liver were snap frozen in liquid nitrogen and stored at −80°C; embedded in OCT compound (VWR, Radnor, PA), frozen, and stored at −80°C; or fixed in Zn-buffered formalin solution (0.1% $ZnSO_4$; 3.8% formaldehyde; VWR) for 2 days, and then placed in 10% and then 30% sucrose solution in PBS for 2 days each on ice. Fixed tissues were kept in 70% ethanol at room temperature until paraffin processing and sectioning at 5 μm.

To determine the amount of steatosis (accumulation of fat in the cells of the liver), OCT-embedded unfixed liver tissue sections were stained with oil red O to detect the accumulation of lipids, as described elsewhere.[23] Paraffin-embedded tissue sections were stained with hematoxylin and eosin to determine adipocyte size, inflammation, and fibrosis, as described previously.[21,24]

Before antibody staining, fixed tissue sections were deparaffinized, and antigens were retrieved using 10 mmol/L sodium citrate, pH 6.0, at 98°C for 20 minutes, as described earlier.[25] As previously described,[15,21,24] sections of adipose and liver tissue were stained with 5 μg/mL antibodies against Mac2 [rat monoclonal antibody (Ab); clone M3/38; BioLegend, San Diego, CA] to detect inflammatory and tissue macrophages, F4/80 (rabbit monoclonal Ab; D2S9R; Cell Signaling Technology, Danvers, MA) to detect tissue resident macrophages, CD64 (rabbit monoclonal Ab; 50086-R001; SinoBiological, Wayne, PA) to detect Fc-γ receptor 1 expression, C-type lectin domain family 4 member F (CLEC4F; goat Ab; AF2784; Novus Biologicals, Littleton, CO) to specifically detect Kupffer cells, MRP8 (goat Ab; R&D Systems, Minneapolis, MN) to detect neutrophils and inflammatory macrophages, or mitochondrial uncoupling protein 1 (UCP1; rabbit Ab; GTX112784; Genetex, Irvine, CA) found in brown fat. Some adipose and liver sections were incubated overnight at 4°C with 1 μg/mL rabbit polyclonal anti-NEU1 (TA335236; Origene, Rockville, MD), anti-NEU2 (TA324482; Origene), or anti-NEU4 (AP52856PU-N; Origene) in PBS + 2% bovine serum albumin or NEU3 (TA343356; Origene) in PBS containing 500 mmol/L NaCl, 2% bovine serum albumin, and 0.1% NP-40 alternative (EMD Millipore, Billerica, MA), as described previously.[15,16] Secondary F(ab')$_2$ biotin-conjugated donkey anti-rat, anti-goat, or anti-rabbit antibodies were from Jackson ImmunoResearch (West Grove, PA) or Novus Biologicals, and biotinylated antibodies were revealed with streptavidin-conjugated alkaline phosphatase staining (Vector Laboratories, Burlingame, CA). Sections were counterstained with hematoxylin.

Stromal Vesicular Cell Culture

Stromal vascular fraction (SVF) cell isolation from epididymal white adipose tissue of C57BL/6 mice on the regular diet was performed as described.[21,26] SVF cells were resuspended at 5×10^4 cells/mL in Dulbecco's modified

Eagle's medium (Lonza, Wakersville, MD), containing 10% calf serum (Seradigm-VWR), 100 U/mL penicillin, 100 µg/mL streptomycin, and 2 mmol/L glutamine (Lonza) at 37°C in a humidified 5% CO_2 incubator in 96-well µ-plates (ibidi, Madison, WI). At approximately 80% confluence, differentiation was induced by incubating cells in Dulbecco's modified Eagle's medium containing serum, antibiotics, 250 ng/mL dexamethasone, 500 ng/mL insulin, and 40 µg/mL 3-isobutyl-1-methylxanthine (all from MilliporeSigma), in the presence or absence of 200 µmol/L DANA. After 4 days, the cells were air dried, fixed, and stained with oil red O or anti-perilipin antibodies (Novus Biologicals), as described previously.[21,27]

Image Quantification

Tissue sections stained with antibodies, hematoxylin and eosin, or oil red O were imaged with a Nikon Eclipse Ti2 microscope (Nikon Instruments, Melville, NY) and analyzed with ImageJ2 software Fiji version 1.53d (NIH, Bethesda, MD; https://imagej.net/Welcome, last accessed August 30, 2020).[28] The percentage area of stained tissue was quantified as a percentage of the total area of the tissue, as described previously.[15,21,24,29] Adipocyte size was calculated using the ImageJ plug-in Adiposoft version 1.16 (Imaging Unit of the Center for Applied Medical Research, University of Navarra, Pamplona, Spain).[30]

Cytokine and Serum Protein Quantification

After mice were euthanized, blood was collected from the abdominal aorta and chilled on ice. After 30 minutes, the blood was clarified by centrifugation at $10,000 \times g$ for 5 minutes at 4°C to isolate serum, which was then stored at −80°C. Serum cytokines were measured with a 13-plex LEGENDplex Mouse Inflammation Panel kit (BioLegend) following the manufacturer's instructions using an Accuri C6 flow cytometer (Accuri C6; BD Biosciences, San Jose, CA). Data were analyzed using LEGENDplex data analysis software version 8.0 (BioLegend), and the concentration of proteins was calculated from standard curves. Alanine transaminase (ALT) and aspartate aminotransferase (AST) levels were measured by enzyme activity assays (Cayman Chemical, Ann Arbor, MI), following the manufacturer's instructions.

Statistical Analysis

Statistical analysis was performed using Prism software version 7.05 (GraphPad Software, La Jolla, CA). Statistical significance between two groups was determined by t-test, or between multiple groups using analysis of variance with the Sidak test post-test, and significance was defined as $P < 0.05$.

Results

DANA, but Not Loss of NEU3, Reduces High-Fat Diet—Induced Increases in Body and Organ Weights

Obesity is linked with inflammation in adipose tissue and liver.[8,31] Previous research has found that injections of DANA inhibit bleomycin-induced lung inflammation and fibrosis,[15] which is reduced in Neu3 knockout mice ($Neu3^{−/−}$).[16] To determine if DANA can inhibit HFD-induced weight gain, adipose tissue or liver inflammation, or steatosis, mice on an HFD were treated with or without injections of DANA. Compared with C57BL/6 mice on a regular diet, C57BL/6 mice on the HFD had significant weight gain (Figure 1A). Mice fed regular chow and treated with either PBS or DANA injections, on the other hand, had no significant differences in weight (Figure 1A). In the C57BL/6 HFD-fed mice, compared with the PBS-injected mice, there was a significant reduction in weight gain observed in mice treated with DANA injections starting at 21 days of DANA treatment (Figure 1A). To determine whether DANA could prevent HFD-induced increases in organ weights, mice were euthanized and epididymal white adipose and interscapular brown adipose tissue, liver, spleen, lungs, and kidneys were weighed at day 35. Mice fed regular rodent chow and treated with either DANA or PBS injections had no significant differences in any organ weights (Figure 1C and Supplemental Figure S1). As a percentage of body weight, HFD-fed mice had higher white and brown adipose tissue weights compared with mice on regular chow. HFD-fed mice treated with DANA injections had lower white fat and brown fat weights compared with control HFD-fed mice (Figure 1C). As a percentage of body weight, C57BL/6 mice on the HFD had lower liver weights compared with mice on regular chow, although the actual liver weights were slightly higher in HFD-fed mice (means ± SEM, 1.97 ± 0.2 g; $n = 5$), compared with those fed on regular chow (1.75 ± 0.07 g; $n = 5$). HFD-fed mice treated with DANA had higher liver weights compared with control HFD-fed mice (Figure 1C).

There was no significant difference in HFD-induced weight gain between C57BL/6 and $Neu3^{−/−}$ mice (Figure 1B), suggesting that there appears to be no general inability of $Neu3^{−/−}$ mice to process food and increase weight. Compared with $Neu3^{−/−}$ mice fed regular rodent chow, HFD-fed $Neu3^{−/−}$ mice had higher white and brown fat weights, and lower liver tissue weights. Compared with C57BL/6 mice, $Neu3^{−/−}$ mice fed regular chow had significantly higher liver weights. There was no significant difference in adipose or liver weights between C57BL/6 and $Neu3^{−/−}$ mice fed HFD (Figure 1C). These data indicate that injections of DANA can modulate organ weights in diet-induced obesity. However, NEU3 may not be involved with HFD-induced weight gain, and the effects of DANA on body and organ weights may not be due to DANA inhibiting NEU3.
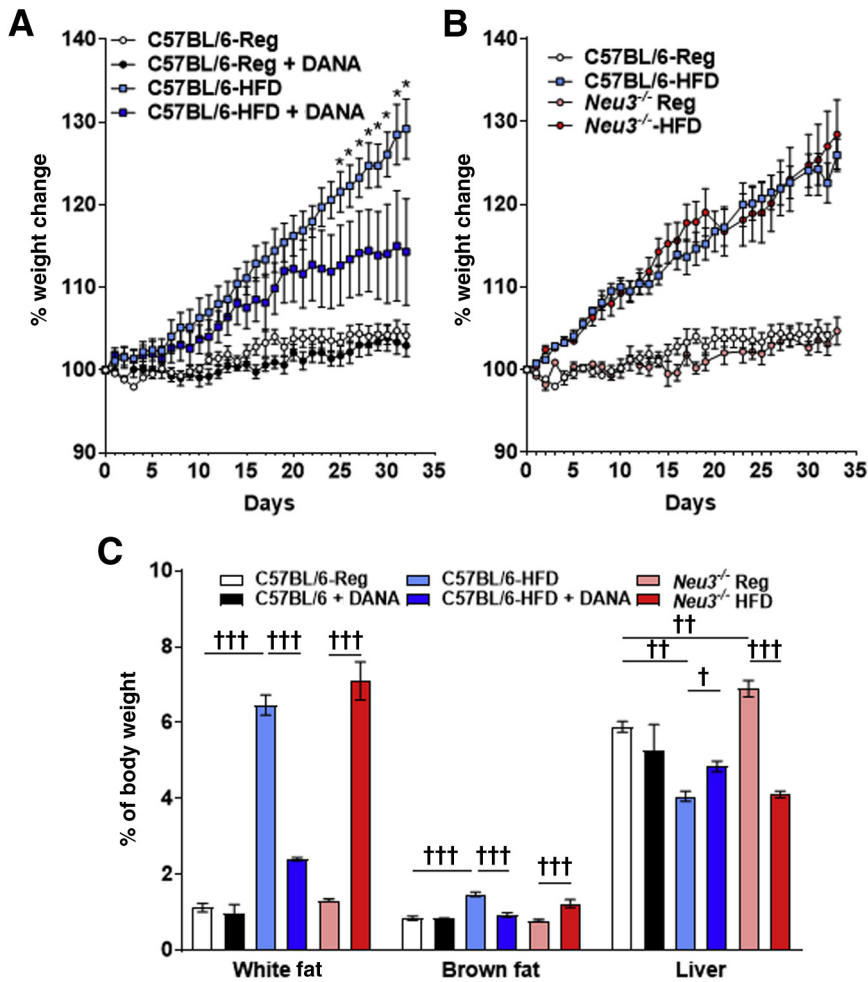
Figure 1 N-acetyl-2,3-dehydro-2-deoxyneuraminic acid (DANA)—treated mice are resistant to high-fat diet (HFD)—induced increases in total body and organ weight. C57BL/6 or *Neu3*$^{-/-}$ mice were maintained on regular diet (Reg) or HFD for 6 weeks before the start of treatment. Mice were injected every 48 hours with phosphate-buffered saline or DANA for 35 days. **A** and **B:** Graphs show body weights of C57BL/6 (**A**) and *Neu3*$^{-/-}$ (**B**) mice. **C:** Epididymal white fat, brown fat, and liver weights. Data are expressed as means ± SEM. *n* = 3 to 6 mice per group. *$P < 0.05$ comparing C57BL/6 mice on HFD and mice on HFD and injected with DANA at each day, significant from day 24 through day 33 (*t*-test); $^{†}P < 0.05$, $^{††}P < 0.01$, and $^{†††}P < 0.001$ (one-way analysis of variance, Sidak test).

DANA, but Not Loss of NEU3, Attenuates an HFD-Induced Increase in Glucose Levels after Glucose Injection

Obesity leads to systemic metabolic dysregulation and type 2 diabetes, with an inability to effectively regulate systemic glucose levels.[8,32] As seen previously,[33,34] compared with C57BL/6 mice on a regular diet, HFD-fed mice had a significant increase in basal glucose levels (Figure 2A). Mice fed standard rodent chow and treated with either DANA or PBS injections had no significant differences in fasting glucose levels (time = 0 minutes) or glucose levels after the glucose injection (Figure 2A). HFD-fed mice injected with DANA had significantly lower blood glucose levels at 60 minutes compared with control HFD-fed mice (Figure 2A), and using area under the curve analysis, HFD-fed mice treated with DANA had significantly lower total glucose levels compared with the HFD-fed control group (Figure 2C).

Neu3$^{-/-}$ mice on regular diet had similar fasting glucose levels to C57BL/6 mice (Figure 2B). Compared with *Neu3*$^{-/-}$ mice on a regular diet, HFD-fed *Neu3*$^{-/-}$ mice had

a significant increase in glucose levels, both at time = 0 and after the glucose injection (Figure 2, B and C). There was no difference in blood glucose levels following glucose injection between C57BL/6 and *Neu3*$^{-/-}$ mice on regular or HFD (Figure 2, B and C). The results suggest that DANA injections may have some ability to regulate glucose levels in obesity, and that this may be due to a NEU3-independent process.

DANA or Loss of NEU3 Decreases Crown-Like Structures in White Adipose Tissue in Mice on an HFD

Obesity leads to an increase in adipocyte size (hypertrophy) and/or an increase in adipocyte numbers (hyperplasia).[34,35] Compared with adipocytes in the white adipose tissue from C57BL/6 and *Neu3*$^{-/-}$ mice on the regular diet (Figure 3, A, E and G, and Supplemental Figure S2), adipocytes in C57BL/6 and *Neu3*$^{-/-}$ mice on the HFD were larger (Figure 3, C, F, and G, and Supplemental Figure S2). Compared with HFD buffer-treated mice, DANA-treated C57BL/6 mice had a significant reduction in adipocyte size (Figure 3, A–D and G, and Supplemental Figure S2).
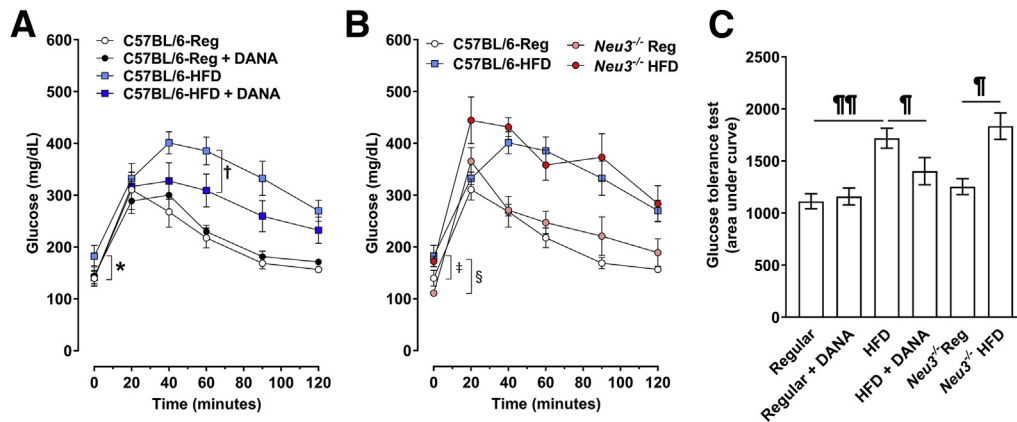
Figure 2 N-acetyl-2,3-dehydro-2-deoxyneuraminic acid (DANA)—treated mice have improved glucose tolerance. **A** and **B:** C57BL/6 mice on regular diet (Reg) or high-fat diet (HFD) for 6 weeks before the start of treatment. Mice were injected every 48 hours with phosphate-buffered saline or DANA. $Neu3^{-/-}$ mice on Reg or HFD did not receive injections. At 35 days, mice were fasted overnight and then received an i.p. injection of glucose (1.5 g/kg body weight), and tail vein blood samples were assessed for glucose at the indicated times. **C:** Glucose tolerance over 120 minutes was assessed by area under the curve analysis. Data are expressed as means $\pm$ SEM. *$P < 0.05$ comparing mice on Reg and HFD (t-test); †$P < 0.05$ comparing mice on HFD and HFD + DANA (t-test); ‡$P < 0.05$ comparing mice on Reg and HFD (t-test); §$P < 0.05$ comparing $Neu3^{-/-}$ mice on Reg and HFD; ¶$P < 0.05$, ¶¶$P < 0.01$ (one-way analysis of variance, Sidak test).

There was no significant difference in adipocyte size between C57BL/6 and $Neu3^{-/-}$ mice fed regular or HFD (Figure 3G and Supplemental Figure S2). These data suggest that injections of DANA, but not disruption of Neu3, can decrease white adipose tissue adipocyte cell size in mice on an HFD.

Although adipose tissue from nonobese mammals contains a variety of tissue-resident immune cells, the accumulation of inflammatory cells in obesity may contribute to both local adipocyte tissue dysfunction and drive systemic inflammation.[8,33] To determine if DANA and/or NEU3 could regulate the number or composition of adipose tissue immune cells, the current study assessed cells in tissue sections. Mac2-positive macrophages that aggregate into crown-like structures (CLSs) are thought to scavenge lipid droplets and dead adipocytes, and the number of
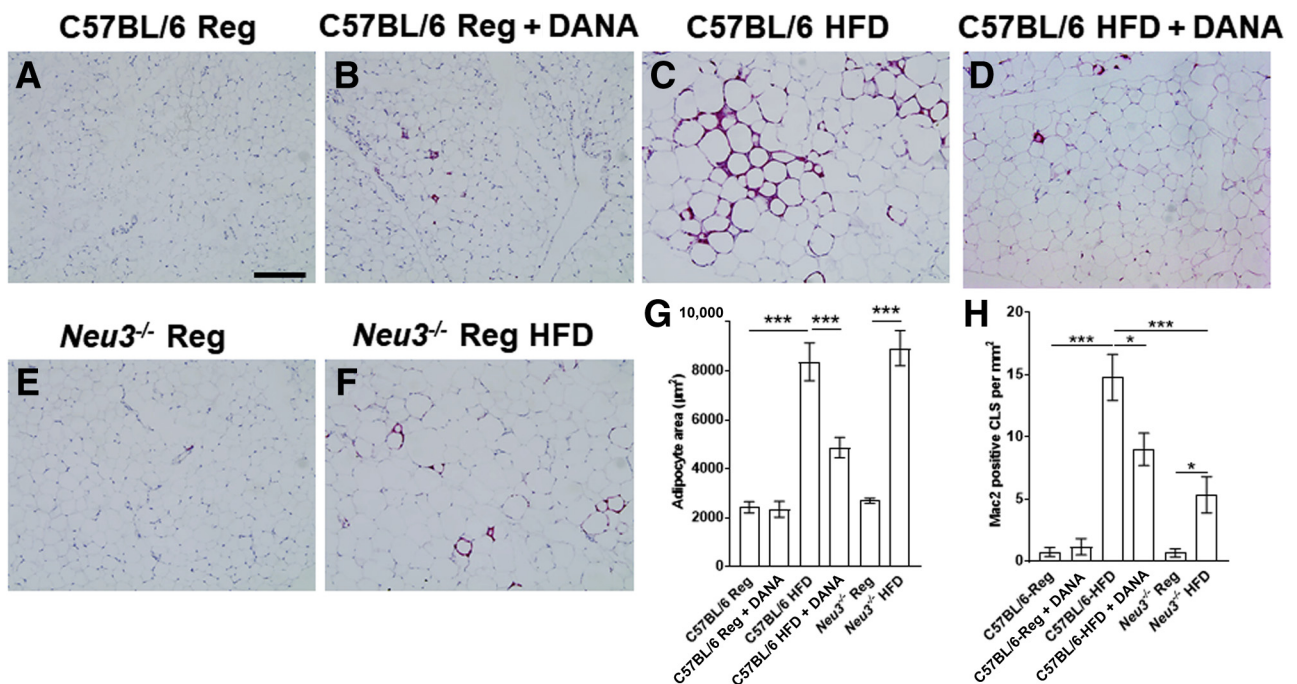

Figure 3 N-acetyl-2,3-dehydro-2-deoxyneuraminic acid (DANA) injections and loss of neuraminidase 3 (NEU3) reduce high-fat diet (HFD)—induced inflammation in white adipose tissue. **A—F:** Representative images of epididymal white fat sections of C57BL/6 mice (**A—D**) or $Neu3^{-/-}$ mice (**E** and **F**) on regular diet (Reg; **A** and **E**), Reg + DANA (**B**), HFD (**C** and **F**), or HFD + DANA (**D**) were stained with anti-Mac2 antibodies. Images are representative of three to eight mice per condition. **G:** Average adipocyte area was calculated. **H:** Mac2-positive crown-like structures (CLSs) were counted. Data are expressed as means $\pm$ SEM (**G** and **H**). $n = 3$ to 8 mice per group (**G** and **H**). *$P < 0.05$, ***$P < 0.001$ (one-way analysis of variance, Sidak test). Scale bar = 0.2 mm (**A—F**).

CLSs correlates with systemic insulin resistance in obese patients.[31,36,37] Compared with mice on a regular diet, both C57BL/6 and *Neu3*^{−/−} mice on an HFD had a significant increase in the number of Mac2-positive CLSs (Figure 3, A, C, E, F, and H, and Supplemental Figure S3). Compared with buffer-treated HFD C57BL/6 mice, DANA-treated HFD C57BL/6 mice had a significant reduction in the number of Mac2-positive CLSs (Figure 3, C, D, and H, and Supplemental Figure S3). There was no difference in the number of Mac2-positive CLSs between C57BL/6 mice and *Neu3*^{−/−} mice on the regular diet (Figure 3, A, E, and H, and Supplemental Figure S3). Compared with C57BL/6 mice on the HFD, the number of Mac2-positive CLSs was significantly lower in HFD-fed *Neu3*^{−/−} mice (Figure 3, C, F, and H, and Supplemental Figure S3). These data suggest that injections of DANA or loss of NEU3 decreases macrophage accumulation in white adipose tissue in mice on an HFD.

DANA or Loss of NEU3 Decreases Adipose Tissue Macrophages in Mice on an HFD

Macrophages are the most abundant immune cells in the adipose tissue of humans and mice, and the composition of macrophage populations changes with obesity.[8,33] Tissue-resident adipose macrophages express CD64 and F4/80, whereas inflammatory macrophages express a variety of markers, including Mac2.[38−41] As observed previously for C57BL/6 mice,[40,42,43] compared with normal-diet mice, both C57BL/6 and *Neu3*^{−/−} mice on an HFD had increased numbers of F4/80- and Mac2-positive cells in the adipose tissue, and treatment with DANA significantly reduced these numbers in C57BL/6 mice (Figure 4 and Supplemental Figure S3). For mice on either regular diet or HFD, there were fewer CD64-, F4/80-, and Mac2-positive cells in *Neu3*^{−/−} mice compared with C57BL/6 mice
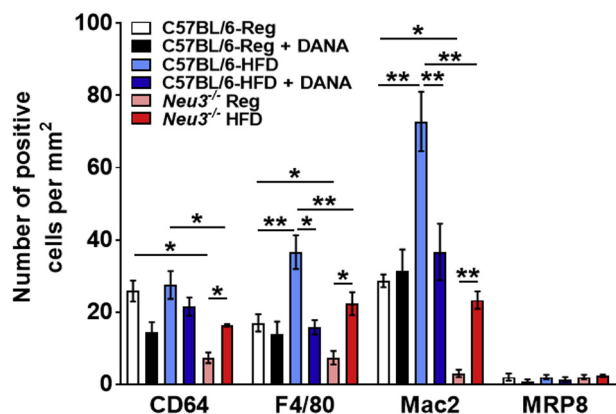

Figure 4 N-acetyl-2,3-dehydro-2-deoxyneuraminic acid (DANA) injections or loss of neuraminidase 3 (NEU3) reduces high-fat diet (HFD)−induced changes in adipose tissue macrophages. Sections of epididymal white adipose tissue were stained with antibodies for the indicated markers. Data are expressed as means ± SEM. *n* = 3 to 8 mice per group. *P < 0.05, **P < 0.01 (one-way analysis of variance, Sidak test). Reg, regular diet.

(Figure 4 and Supplemental Figure S3). To determine if the increase in the number of F4/80- and Mac2-positive cells was indicative of a general inflammatory response, adipose tissues were stained with anti-MRP8 antibodies to detect neutrophils.[44] There were few MRP8-positive cells in white adipose tissue, and no differences were detected in the number of MRP8-positive cells in either C57BL/6 or *Neu3*^{−/−} mice on regular or HFD (Figure 4 and Supplemental Figure S3). These data suggest that HFD-induced increases in white adipose tissue macrophage numbers can be attenuated by injections of DANA, and that loss of NEU3 decreases some HFD-induced inflammation.

DANA Inhibits Adipocyte Differentiation

To determine if DANA can directly regulate adipocyte differentiation, SVF cells were differentiated from regular diet C57BL/6 mice *in vitro* in the presence or absence of DANA, and cells were stained for neutral lipids with oil red O, and for the lipid droplet protein perilipin.[45] Differentiated SVF cells showed positive perilipin and oil red O staining (Figure 5, A, B, and D). Significant inhibition of oil red O−positive SVF cells was observed with increasing concentrations of DANA (Figure 5C). Compared with control differentiated SVF cells, SVF cells differentiated in the presence of DANA had reduced percentages of perilipin-positive cells (Figure 5D). Undifferentiated SVF cells cultured in the presence or absence of DANA had no detectable perilipin staining (Figure 5D). These data suggest that DANA can inhibit adipocyte differentiation.

DANA or Loss of NEU3 Attenuates HFD-Induced Changes in Brown Fat UCP1 Expression

High-fat diet−induced obesity also leads to changes in brown adipose tissue.[8,35] As previously observed,[46] compared with C57BL/6 mice on a regular diet, the brown fat of HFD-fed C57BL/6 mice had a reduced percentage of the brown fat tissue staining for nonfat cytoplasm with hematoxylin and eosin (Figure 6G and Supplemental Figure S4, A and C), also observed in *Neu3*^{−/−} mice (Figure 6G and Supplemental Figure S4, E and F). Compared with C57BL/6 HFD-fed mice, mice treated with DANA had a higher percentage of the brown fat tissue staining for nonfat cytoplasm with hematoxylin and eosin (Figure 6G and Supplemental Figure S4, A−D). Brown fat can process lipids and carbohydrate to generate UCP1-mediated heat (adaptive thermogenesis).[47,48] Compared with control diet-fed mice, there was reduced UCP1 staining of brown fat in HFD-fed C57BL/6 mice, which was reversed by DANA (Figure 6, A−D and H). Although the brown fat of *Neu3*^{−/−} mice on the HFD had a reduced percentage of the brown fat tissue staining for nonfat cytoplasm with hematoxylin and eosin compared with *Neu3*^{−/−} mice on the regular diet, there was no reduction in the UCP1 staining in the tissue (Figure 6, E, F, and H). In
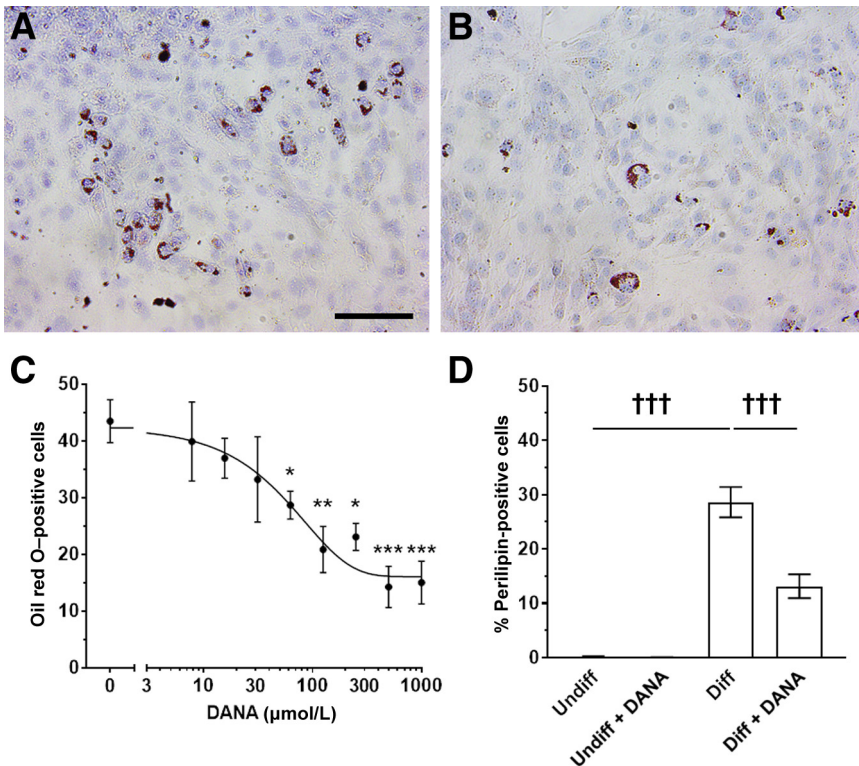
Figure 5 N-acetyl-2,3-dehydro-2-deoxyneura-minic acid (DANA) inhibits adipocyte differentia-tion. **A** and **B:** Stromal vesicular fraction (SVF) cells isolated from C57BL/6 mice on a regular diet were differentiated in the absence (**A**) or presence (**B**) of DANA and were stained with oil red O. Images are representative of three mice per condition. **C:** Quantification of oil red O—positive cells. **D:** Cells were stained with antiperilipin antibodies, and the percentage of cells stained was counted. **C** and **D:** Cells from three mice per group. Data are expressed as means ± SEM (**C** and **D**). *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$ comparing SVF cells in the presence or absence of DANA (t-test); [†††]$P < 0.001$ comparing percentage of cells stained (one-way analysis of variance, Sidak test). Scale bar = 0.1 mm (**A** and **B**). Diff, differentiated; Undiff, undif-ferentiated.

addition, compared with C57BL/6 mice on the HFD, $Neu3^{-/-}$ mice on the HFD had significantly more UCP1 staining (Figure 6H). These data suggest that the accumulation of lipid in the brown fat adipocytes and UCP1 protein levels are independently controlled, with DANA but not loss of NEU3 reducing lipid accumulation and NEU3 regulating UCP1 levels.

DANA and NEU3 Reduce HFD-Induced Changes in Liver Inflammation and Steatosis

Obesity is associated with nonalcoholic fatty liver disease, a spectrum of liver diseases that ranges from simple steatosis (accumulation of fat droplets within the hepatocytes), to hepatitis (inflammation) resulting in nonalcoholic steatosis, and ultimately cirrhosis (fibrosis) and liver failure.[49,50] Obesity is also associated with liver inflammation, especially increase in macrophages.[8,51,52] As observed previously,[8,42] compared with regular-diet mice, C57BL/6 mice on HFD had increased numbers of CD64-, F4/80-, and Mac2-positive cells in the liver (Figure 7, A—C, and Supplemental Figure S5). Injections of DANA reduced the numbers of F4/80- but not CD64- or Mac2-positive cells in C57BL/6 mice fed an HFD (Figure 7, A—C, and Supplemental Figure S5). There were fewer CD64- and Mac2-positive cells in $Neu3^{-/-}$ mice compared with C57BL/6 mice maintained on either regular or HFD (Figure 7, A and C, and Supplemental Figure S5).

Compared with C57BL/6 mice, $Neu3^{-/-}$ mice on a regular diet had similar numbers of F4/80-positive cells, but on an HFD, $Neu3^{-/-}$ mice had a lower density of F4/80 cells in the liver (Figure 7B and Supplemental Figure S5). The number of Mac2-positive cell clusters was significantly higher in both C57BL/6 and $Neu3^{-/-}$ mice fed an HFD, but $Neu3^{-/-}$ mice had fewer Mac2 cell clusters than C57BL/6 mice (Figure 7D and Supplemental Figure S5). No differences were detected in the total number of CLEC4f-positive Kupffer cells[53—55] or MRP8-positive neutrophils between C57BL/6 and $Neu3^{-/-}$ mice, or mice fed regular or HFD (Figure 7, E and F, and Supplemental Figure S5). These data suggest that HFD-induced increases in liver macrophage numbers are attenuated by loss of NEU3 and attenuated for F4/80 macrophages by injections of DANA.

For both C57BL/6 and $Neu3^{-/-}$ mice, compared with mice on the regular diet, mice on the HFD had increased steatosis, increased serum ALT and AST levels, and accumulation of oil red O staining (Figure 8, A, B, E, and F, and Supplemental Figure S6, A—E). Compared with C57BL/6 mice, there were no significant differences between steatosis, accumulation of oil red O staining, or AST levels, but ALT levels were higher in $Neu3^{-/-}$ mice (Figure 8 and Supplemental Figure S6). Compared with HFD-fed control mice, DANA-treated C57BL/6 mice had reduced steatosis and serum ALT and AST levels (Figure 8, C, D, and G, and Supplemental Figure S6, F and G). These data suggest that
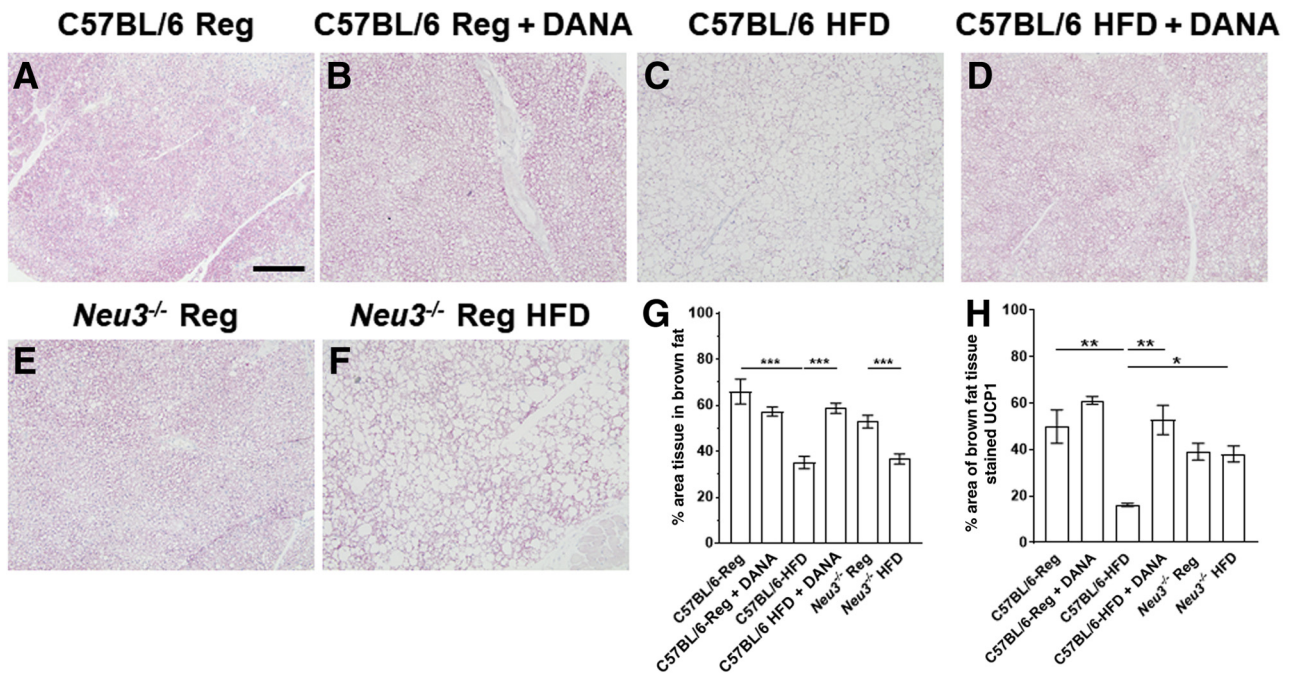
Figure 6 N-acetyl-2,3-dehydro-2-deoxyneuraminic acid (DANA) reduces high-fat diet (HFD)—induced changes in brown adipose tissue. **A**—**F**: C57BL/6 (**A**—**D**) and *Neu3*$^{-/-}$ (**E** and **F**) mice on regular diet (Reg; **A** and **E**), HFD (**C** and **F**), Reg + DANA (**B**), or HFD + DANA (**D**). **A**—**F**: Representative images of brown fat sections were stained with anti—uncoupling protein 1 (UCP1) antibodies. Images are representative of three to seven mice per condition. **G**: Quantification of percentage tissue area that is not fat. **H**: Percentage tissue area stained with anti-UCP1 antibodies. Data are expressed as means ± SEM (**G** and **H**). $n = 3$ to 7 mice per group (**G** and **H**). *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$ (one-way analysis of variance, Sidak test). Scale bar = 0.2 mm (**A**—**F**).

DANA, but not loss of NEU3, can reverse some of the effects of high-fat diet on liver steatosis.

Diet-induced obesity leads to the production of inflammatory cytokines by adipose tissue macrophages, dendritic cells, and T cells.[8] As expected,[8] C57BL/6 mice fed an HFD had elevated serum levels of tumor necrosis factor-α (Supplemental Figure S7). Compared with C57BL/6 mice on HFD, *Neu3*$^{-/-}$ mice had lower serum levels of IL-6, IL-23, tumor necrosis factor-α, and monocyte chemoattractant protein 1, and compared with *Neu3*$^{-/-}$ mice on the regular diet, *Neu3*$^{-/-}$ mice on HFD had reduced IL-23 (Supplemental Figure S7). DANA reduced serum levels of IL-17A (Supplemental Figure S7). These data suggest that DANA and NEU3 can affect some systemic cytokines associated with diet-induced obesity.

Sialidase Expression in Adipose and Liver Tissue

The role of sialidases in the regulation of HFD-induced obesity is unclear, with up-regulation or down-regulation of different sialidase proteins in different tissues, suggesting a complex association between obesity and sialidases.[17−19] To determine the expression of sialidases, adipose and liver sections were stained with antibodies against the four sialidases. The antibodies were made against domains of the sialidases that are different from each other, do not bind the other sialidases, and stain bands of the expected molecular weights on Western blots of whole cell lysates and lung fluid.[15,16] As testicular tissue and blood vessels express sialidases,[56,57] sections of white fat containing testicular tissue or blood vessels, or brown fat with adjacent white fat were stained to further validate these antibodies and to show the differential expression of sialidases between these tissues (Supplemental Figure S8). Staining for NEU1, NEU2, and NEU3 was observed in both testicular tissue and blood vessels, and staining was more pronounced in brown fat than in white fat (Supplemental Figure S8). NEU4 is expressed predominantly in neuronal tissue,[58] and weak NEU4 staining was observed in all tissues tested (Supplemental Figure S8). For both white and brown fat, the staining of all four sialidases was not significantly different between regular and HFD-fed C57BL/6 mice, but DANA injections reduced staining for NEU3 in adipose tissue in mice on regular diet (Figure 9, A and B, and Supplemental Figures S9 and S10). There were no significant differences in the staining for NEU1 between C57BL/6 and *Neu3*$^{-/-}$ mice on the regular or high-fat diet (Figure 9, A and B, and Supplemental Figures S9 and S10). Compared with C57BL/6 mice, NEU2 and NEU4 staining in white fat was reduced in *Neu3*$^{-/-}$ mice fed either regular or HFD, and NEU2 and NEU4 staining in brown fat was reduced in HFD fed *Neu3*$^{-/-}$ mice (Figure 9, A and B, and Supplemental Figures S9 and S10). In the liver, DANA increased NEU1
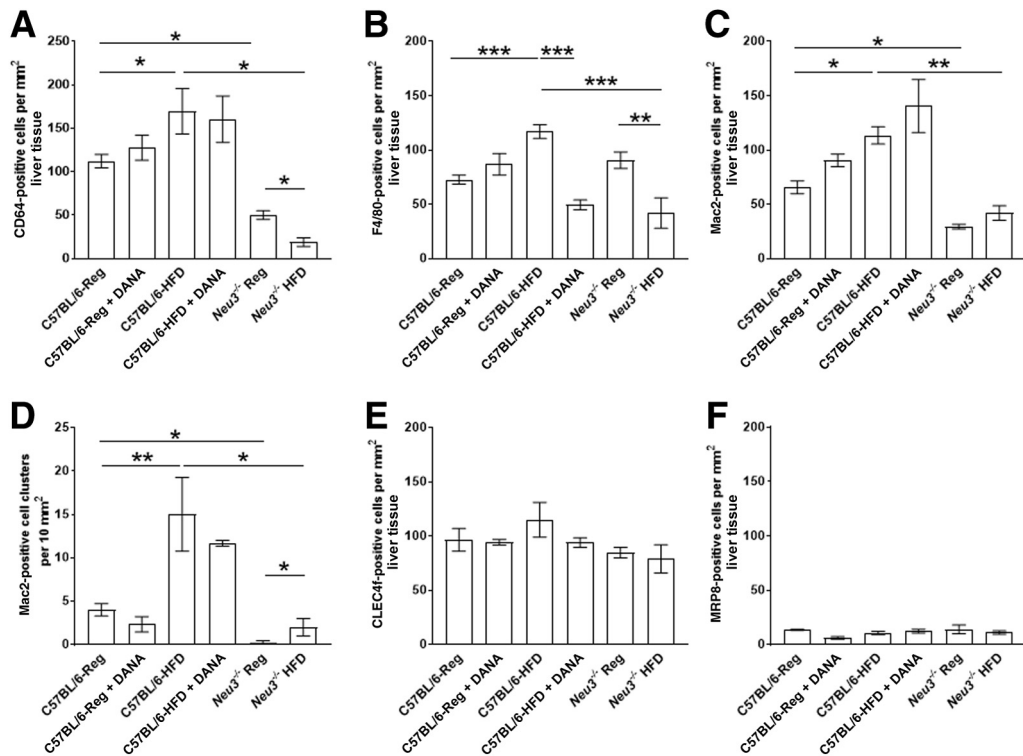
Figure 7 N-acetyl-2,3-dehydro-2-deoxyneuraminic acid (DANA) injections or loss of NEU, neuraminidase 3 (NEU3) reduces high-fat diet (HFD)—induced changes in liver macrophages. Sections of liver tissue were stained with antibodies for CD64 (**A**), F4/80 (**B**), Mac2 (**C**), Mac2 cell clusters (**D**), C-type lectin domain family 4 member F (CLEC4f; **E**), and MRP8 (**F**). Data are expressed as means ± SEM. $n = 3$ to 8 mice per group. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$ (one-way analysis of variance, Sidak test). Reg, regular diet.

and NEU3 staining in C57BL/6 mice, and reduced NEU3 staining in HFD-fed mice (Figure 9C and Supplemental Figure S11). Staining for NEU1, NEU2, and NEU4 was almost undetectable in liver tissue from $Neu3^{-/-}$ mice

(Figure 9C and Supplemental Figure S11). These data suggest that DANA can reverse some of the effects of high-fat diet independently of sialidase levels in the adipose and liver tissues.
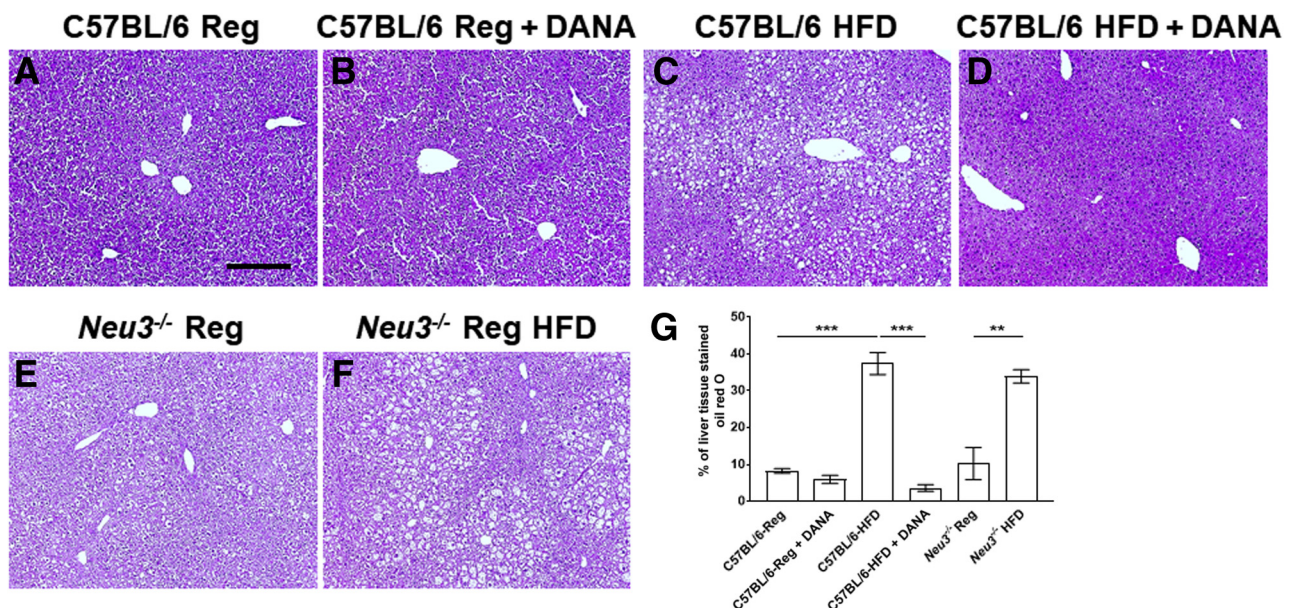

Figure 8 N-acetyl-2,3-dehydro-2-deoxyneuraminic acid (DANA) injections reduce high-fat diet (HFD)—induced changes in liver steatosis. **A**—**F**: C57BL/6 (**A**—**D**) and $Neu3^{-/-}$ (**E** and **F**) mice on regular diet (Reg; **A** and **E**), Reg + DANA (**B**), HFD (**C** and **F**), and HFD + DANA (**D**). Images show representative liver sections stained with hematoxylin and eosin. **G:** Quantification of liver sections stained with oil red O stain. Data are expressed as means ± SEM (**G**). $n = 3$ to 8 mice per group (**G**). **$P < 0.01$, ***$P < 0.001$ (one-way analysis of variance, Sidak test). Scale bar = 0.2 mm (**A**—**F**).
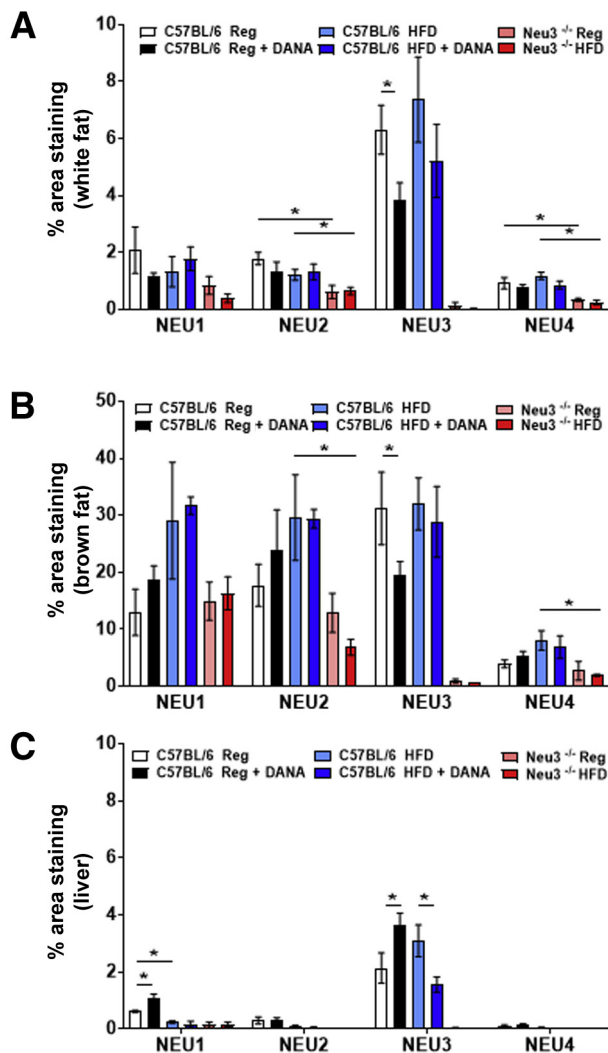
Figure 9 N-acetyl-2,3-dehydro-2-deoxyneuraminic acid (DANA) injections or loss of neuraminidase (NEU) 3 reduces high-fat diet (HFD)—induced changes in sialidase expression. Sections of white fat (**A**), brown fat (**B**), or liver (**C**) were stained with antibodies against NEU1, NEU2, NEU3, and NEU4. Graphs show quantification of percentage tissue area stained with antibodies. Data are expressed as means ± SEM. $n = 3$ to 4 mice per group. *$P < 0.05$ (one-way analysis of variance, Sidak test). Reg, regular diet.

Discussion

Both injections of DANA and loss of NEU3 were found to improve multiple features of high-fat diet—induced obesity in mice. Injections of the general sialidase inhibitor DANA moderate weight gain, attenuate elevated glucose levels, reduce liver steatosis, and decrease adipose and liver inflammation. Compared with C57BL/6 mice on HFD, $Neu3^{-/-}$ mice had reduced HFD-induced adipose and liver inflammation. These data suggest that DANA can decrease adipose tissue and liver inflammation, and $Neu3^{-/-}$ mice are resistant to HFD-induced adipose tissue and liver inflammation. The observation that DANA can

inhibit weight gain and reduce steatosis, whereas $Neu3^{-/-}$ mice are not resistant to HFD-induced weight gain and steatosis, indicates that DANA acts on more than one sialidase, whereas the genetic loss of NEU3 indicates that this enzyme is important for regulating inflammation. Both DANA and loss of NEU3 reduced the numbers of Mac2-positive macrophages that aggregate into crown-like structures and tissue-resident F4/80-positive macrophages. There were fewer CD64-, F4/80-, and Mac2-positive cells present in the adipose tissue of $Neu3^{-/-}$ mice on either regular or HFD, compared with C57BL/6 mice. DANA also reduced the accumulation of lipids in adipocyte cultures, and both DANA and loss of NEU3 inhibited the accumulation of lipids in brown adipose tissue. DANA and loss of NEU3 also attenuated HFD-induced changes in brown fat UCP1 expression. In the liver, both DANA and loss of NEU3 reduced the HFD-induced increase in F4/80-positive but not CD64- and Mac2-positive macrophages. DANA, but not NEU3, also reduced liver steatosis. The effects of DANA and loss of NEU3 appear to be mostly independent of serum ALT, AST, and cytokines, and the levels of sialidase proteins in the adipose and liver tissues.

The role of sialidases in the regulation of HFD-induced changes in physiology and immunology is unclear. Compared with nonobese control mice (db/+), obese (db/db) mice on a regular diet have reduced *NEU1* mRNA levels and reduced NEU1 enzyme activity in liver tissue, but increased NEU1 enzyme activity in epididymal white fat.[17] Compared with lean (Fa/fa) Zucker rats, obese (fa/fa) Zucker rats (which develop obesity without diabetes) on a regular diet have reduced NEU3 protein content in the soleus muscle and liver.[18] Wistar rats fed a high-fat diet also have reduced NEU3 protein levels in white fat compared with regular diet controls.[18] Compared with nonobese humans, obese patients have higher *NEU3* mRNA in the small intestine. NEU3 protein was also increased in the small intestines of HFD-treated mice compared with regular diet mice.[59] In addition, oral administration of the sialidase inhibitor DANA to mice inhibited NEU3 enzyme activity in the small intestine, but not in the liver and white adipose tissue, and attenuated hepatic steatosis and HFD-induced weight gain.[59] NEU3 overexpression in the liver using adenovirus improved glucose tolerance and insulin sensitivity, but increased hepatic triglyceride and glycogen accumulation and induced hyperlipidemia.[19] These data indicate that DANA and loss of NEU3 also have a complex effect on sialidase protein levels, indicating that other systems may also be altered by DANA or loss of NEU3. Together, these results suggest a complex association between obesity and sialidases.

Altered sialidase levels can lead to altered insulin signaling.[18,19,60−62] NEU1 can desialylate both insulin and insulin-like growth factor 1 receptors, leading to increased insulin receptor signaling.[61−63] Long-term

hepatic NEU1 overexpression increases glucose intolerance and insulin resistance in mice on an HFD.[63] Reduced liver NEU3 protein levels lead to reduced insulin signaling,[18] whereas increased NEU3 protein in the liver increases insulin sensitivity and glucose tolerance in mice.[19,64] These data suggest that the dysregulation of insulin, glucose, lipids, and inflammation in obesity may be further impacted by changes in sialidase expression and activity.

Obesity is also associated with inflammation, especially the accumulation of macrophages.[8,31,51,52] Both DANA and loss of NEU3 leads to a lower density of Mac2- and F4/80-positive macrophages in both adipose tissue and liver. Under steady-state conditions, tissue-resident adipose macrophages express CD64 and F4/80, whereas inflammatory macrophages express Mac2.[38–41] The observation that HFD-induced increases in F4/80- and Mac2-positive cells in adipose tissue were reversed by DANA suggests that sialidases affect adipose tissue inflammation. Compared with control mice, $Neu3^{-/-}$ mice had significantly lower numbers of CD64-, F4/80-, and Mac2-positive cells in white adipose tissue, even on a regular diet. However, no difference in lung macrophage populations was found between control and $Neu3^{-/-}$ mice.[16] A possible explanation points to the differential expression of sialidases in the two tissues.[58] These data suggest that NEU3 has different effects in different tissues, and affects adipose tissue monocyte/macrophage biology.

The liver also has tissue-resident and inflammatory macrophages.[53,54,65,66] As expected, HFD led to an increase in macrophage numbers in the liver, but DANA only decreased F4/80$^+$ resident, but not CD64$^+$ resident or Mac2$^+$ inflammatory macrophages. Compared with C57BL/6 mice, $Neu3^{-/-}$ mice had significantly lower numbers of CD64- and Mac2-, but not F4/80-positive cells in the liver on regular and high-fat diets. These observations suggest that sialidases regulate at least some of the five recognized liver macrophage populations.[53] Together, these data suggest that one function of sialidases, especially NEU3, is to regulate adipose and liver tissue inflammation, and that sialidase inhibitors are potential therapeutics for high-fat diet−induced inflammation.

Acknowledgments

Supplemental Data

Supplemental material for this article can be found at http://doi.org/10.1016/j.ajpath.2020.09.011.

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