Arteriosclerosis, Thrombosis, and Vascular Biology

BASIC SCIENCES

Dietary Neu5Ac Intervention Protects Against Atherosclerosis Associated With Human-Like Neu5Gc Loss—Brief Report

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OBJECTIVE: Species-specific pseudogenization of the *CMAH* gene during human evolution eliminated common mammalian sialic acid *N*-glycolylneuraminic acid (Neu5Gc) biosynthesis from its precursor *N*-acetylneuraminic acid (Neu5Ac). With metabolic nonhuman Neu5Gc incorporation into endothelia from red meat, the major dietary source, anti-Neu5Gc antibodies appeared. Human-like *Ldlr*$^{-/-}$*Cmah*$^{-/-}$ mice on a high-fat diet supplemented with a Neu5Gc-enriched mucin, to mimic human red meat consumption, suffered increased atherosclerosis if human-like anti-Neu5Gc antibodies were elicited.

APPROACH AND RESULTS: We now ask whether interventional Neu5Ac feeding attenuates metabolically incorporated Neu5Gc-mediated inflammatory acceleration of atherogenesis in this *Cmah*$^{-/-}$*Ldlr*$^{-/-}$ model system. Switching to a Neu5Gc-free high-fat diet or adding a 5-fold excess of Collocalia mucoid-derived Neu5Ac in high-fat diet protects against accelerated atherosclerosis. Switching completely from a Neu5Gc-rich to a Neu5Ac-rich diet further reduces severity. Remarkably, feeding Neu5Ac-enriched high-fat diet alone has a substantial intrinsic protective effect against atherosclerosis in *Ldlr*$^{-/-}$ mice even in the absence of dietary Neu5Gc but only in the human-like *Cmah*-null background.

CONCLUSIONS: Interventional Neu5Ac feeding can mitigate or prevent the red meat/Neu5Gc-mediated increased risk for atherosclerosis, and has an intrinsic protective effect, even in the absence of Neu5Gc feeding. These findings suggest that similar interventions should be tried in humans and that Neu5Ac-enriched diets alone should also be investigated further.

GRAPHIC ABSTRACT: A graphic abstract is available for this article.

Key Words: atherosclerosis ■ inflammation ■ mice ■ mucins ■ *N*-acetylneuraminic acid

Sialic acids (Sias) are a family of nine-carbon-backbone monosaccharides prominently displayed on glycans attached to most cell surface and secreted molecules of all vertebrate cells.[1–5] In keeping with their location, prominence, and extensive structural diversity, Sias mediate or modulate a wide variety of biological processes in health and disease.[5–10] The most common Sia in mammals is *N*-acetylneuraminic acid (Neu5Ac), which is often converted into the next most common type, *N*-glycolylneuraminic acid (Neu5Gc). Independent inactivation events of the *CMAH* gene encoding the CMAH enzyme that mediates conversion of Neu5Ac to Neu5Gc (by addition of a single oxygen atom) have occurred in certain vertebrate taxa, including humans and birds.[11–13] However, metabolic incorporation of diet-derived Neu5Gc occurs in humans who consume red meat (the richest common source of dietary Neu5Gc), resulting in endogenous cell surface display of small amounts of Neu5Gc-glycans in endothelia and some epithelia. These incorporated Neu5Gc-glycans then act as xeno-autoantigens that can interact with circulating anti-Neu5Gc-glycan xeno-autoantibodies, generating a novel diet-mediated inflammatory process called xenosialitis.[14–18]

Our prior work in human-like Neu5Gc-deficient *Cmah*-null mice showed that this inflammatory xenosialitis process accelerates progression of carcinomas[19] and of

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Nonstandard Abbreviations and Acronyms

ABC	ATP-binding cassette transporter
EBN	Edible bird's nest
HFD	high-fat diet
LDL	low-density lipoprotein
Neu1	neuraminidase 1
Neu5Ac	*N*-acetylneuraminic acid
Neu5Gc	*N*-glycolylneuraminic acid
PSM	porcine submaxillary mucin
Sias	sialic acid

Highlights

- Human-specific loss of sialic acid *N*-glycolylneuraminic acid production and associated anti–*N*-glycolylneuraminic acid–glycan antibody generation interact against metabolically incorporated *N*-glycolylneuraminic acid–bearing glycans (enriched in red meat), inducing inflammatory xenosialitis and enhanced atherogenesis.
- Interventional feeding with the *N*-glycolylneuraminic acid precursor *N*-acetylneuraminic acid prevents the xenosialitis-mediated increase in atherogenesis.
- Feeding *N*-acetylneuraminic acid–rich mucins in a humanized atherosclerosis model has strong intrinsic atheroprotective effects independent of xenosialitis.

atherosclerosis,[20] likely contributing toward the human-specific association of these diseases with consumption of red meat (particularly processed red meats, which may perhaps have more bioavailable Neu5Gc).[21,22] Independent of this extrinsic mechanism, we also found that the human-like Neu5Gc-deficient *Cmah*-null mice already had an intrinsic propensity to develop severe aortic atherosclerotic plaques, via multiple mechanisms, including an augmented immune reactivity.[20] We suggested that these factors contribute to the unusual propensity of humans to develop severe atherosclerotic cardiovascular disease,[23–25] which now accounts for one-third of deaths worldwide.

Collocalia mucoid has long been known to biochemists as a rich source of Neu5Ac (≈9% w/w)[26] and is the primary component of Edible bird's nest (EBN)—a popular but expensive Chinese health food, often referred to as the Caviar of the East.[27] The nests are highly enriched in salivary mucins of the White-nest (*Aerodramus fuciphogus*) or Black-nest Swiftlet (*Aerodramus maximus*).[27,28] The high content of Neu5Ac is not surprising, as salivary mucins are rich in Sias (indeed, sialic acid is derived from the word saliva).[29] In the present study, we consider the possibility that some of the claimed health benefits of EBN may be related to its rich content of mucin-associated Neu5Ac (the rest of the composition of EBN consists of common neutral monosaccharides and amino acids).[30] We tested and confirmed that dietary intervention with EBN can attenuate both the intrinsic and extrinsic factors that drive accelerated atherosclerosis development associated with the human-like Neu5Gc deficiency in *Ldlr*$^{-/-}$*Cmah*$^{-/-}$ mice. The study also addresses potential implications for the preservation of the Chinese swiftlet species, which are under ecological pressure due to the increased commercial activity with the harvesting of their nests.[31–33]

MATERIALS AND METHODS

Ethics Statement

The use of mice in this project was approved by the University of California, San Diego (evaluation of the role of glycans in normal physiology, malignancies, and immune responses; protocol S01227). All procedures were approved by the Animal Care Program and Institutional Animal Care and Use Committee, University of California, San Diego.

Mice and Cell Culture

Cmah$^{-/-}$*Ldlr*$^{-/-}$ mice were generated by crossing *Cmah*$^{-/-}$ mice[34] and *Ldlr*$^{-/-}$ mice[35] in a congenic C57BL/6 N background and maintained in the University of California, San Diego vivarium according to the institutional review board guidelines. All animals were fully backcrossed and maintained on a 12-hour light cycle and fed ad libitum with water and standard rodent diet. Recommendations for atherosclerosis studies per the American Heart Association statement were followed,[36] expect for high-fat diet (HFD) composition to allow us to evaluate the impact of dietary Sias sources on atherogenesis. *Cmah*$^{-/-}$*Ldlr*$^{-/-}$ and *Cmah*$^{+/+}$*Ldlr*$^{-/-}$mice were also maintained on a control soy-based (Sia free) diet after weaning at 3 weeks. At 6 or 9 weeks of age, male and female mice were placed on a Sia-free soy-based HFD with 20% anhydrous milk fat and 0.2% cholesterol, a Neu5Ac-rich soy-based HFD (containing 0.25 mg of Neu5Ac per gram of diet; made by adding EBN; Golden Nest, Inc), and a Neu5Gc-rich soy-based HFD (containing 0.25 mg of Neu5Gc per gram of diet, made by adding purified PSM [porcine submaxillary mucin] as described previously).[20] Five-fold Neu5Ac (1.25 mg of Neu5Ac per gram of diet) HFD and a premix of 5-fold Neu5Ac (1.25 mg of Neu5Ac per gram of diet) and regular amount of Neu5Gc (0.25 mg of Neu5Gc per gram of diet) HFD were also added to HFD course (Table I in the Data Supplement). The amount of Neu5Gc in PSM and Neu5Ac in EBN was determined by HPLC with Neu5Ac (Nacalai) and Neu5Gc (Inalco) standards, and all diets were subsequently formulated and composed (Dyets, Inc). A table detailing the contents of the various diets is also provided in the Data Supplement. Peritoneal macrophages were collected by peritoneal lavage after 12 weeks of HFD feeding without any stimulants.[20]

Foam Cell Formation

Human macrophage-like THP-1 cells were cultivated in RPMI 1640 (GIBCO) supplemented with 10% fetal bovine serum (Omega Scientific), 10 mmol/L HEPES buffer (GIBCO), 1

mmol/L sodium pyruvate (GIBCO), 0.25% D-(+)-glucose (Sigma), and 0.05 mmol/L 2-mercaptoethanol, plated in 24-well flat-bottom tissue culture plate at a cell density of 300.000 cells/cm^2 and differentiated into macrophages using 50 ng/mL phorbol 12-myristate 13-acetate (Sigma-Aldrich) for 72 hours. Cells were then washed and cultured with growth media added with 50 µg/mL aggregated LDL cholesterol (Milipore) and incubated for 24 hours with or without 2 mmol/L free Neu5Ac or Neu5Gc feeding.

Neu5Gc Immunization

Chimpanzee or human red blood cell membrane ghosts were prepared as described previously.[17] Six-weeks-old *Cmah*$^{-/-}$*Ldlr*$^{-/-}$ male mice were immunized with pooled red blood cell membrane chimp ghosts, mixed with an equal volume of Freund adjuvant per week via intraperitoneal injection for 3 weeks (using complete adjuvant for week first, then incomplete adjuvant for the second and third weeks).

Serum Lipoprotein and Lipid Analysis

Blood samples were obtained by mandibular plexus bleeding and cardiac puncture from mice fasted for 5 hours. Serum lipoproteins in 100 µL pooled samples were separated by size exclusion chromatography using a polyethylene filter column (Sigma-Aldrich). Liver samples are homogenized in 250 mmol/L sucrose buffer, and the protein levels were measured by the bicinchoninic acid assay (Thermo Fisher Scientific). Cholesterol and triglyceride levels in separated lipoprotein fraction were measured by enzymatic kits (Sekisui, San Diego, CA), as well as total cholesterol and triglyceride levels in serum and liver samples.

Quantification of Aortic Atherosclerosis

Using stereomicroscopy dissection, the heart and ascending aorta of each animal were dissected, all the way down to the iliac bifurcation. The aortas were then opened along the long axis, pinned flat, and stained for lipids using Sudan IV stain. Serial 10-µm cryosections of the aortic sinus were stained with Masson trichrome to measure area under the curve, as well as mean lesion sizes of each of the atherosclerosis plaques, and also to assess the area comprising the necrotic core.[20] Each parameter was calculated using Image J, in a blinded manner, by K.K. and C.D.

Quantification of Inflammatory Cytokine Gene Expression

Cells were collected after completion of feeding regimen, and mRNA was collected using a purification kit and converted into cDNA (Qiagen, Inc). Expression of each cytokine gene (Table II in the Data Supplement) was measured using Cyber Green systems (Qiagen, Inc).

HPLC Quantification of Sias Content by DMB

The ethanol fraction from the macrophage LDL uptake assay was incubated at −80 °C for >3 hours and then lyophilized with a Labconco FreeZone Plus 4.5 L Cascade Benchtop Freeze Dry System (catalog No. 7386030). Samples were acid hydrolyzed with glacial acetic acid (2M final in total volume 200 µL) for 3 hours at 80 °C to remove terminal Sias. Samples were spin-filtered (Millipore Sigma Amicon Ultra-0.5 Centrifugal Filter Unit; cat No. UFC5010BK) to remove cellular debris. Free Sias were then derivatized with DMB as described previously.[37] Sias were quantified with HPLC fluorometry on a Phenomenex C-18 column using isocratic elution in 85% water, 7% methanol, and 8% acetonitrile.

Immunohistochemistry

All aorta tissue samples were fixed and processed for paraffin sections and were stained with routine hematoxylin and eosin, Masson trichrome, or serial cross sections of the aortic sinus were used for immunohistochemistry using the macrophage marker anti-CD68 (Abcam) with nuclei being counterstained with Mayer hematoxylin. Stained samples were photographed using Keyence BZ-9000, and the digital photomicrographs were analyzed using the Image J software.

Statistical Analysis

Statistical analyses were performed using the Prism software (version 9; GraphPad Software). Data are presented as mean (SD) or SEM as indicated. Data have been analyzed for normality and equal variance using GraphPad software. When met, this was a justification for using parametric analysis including Student *t* test, 1-way ANOVA (>2 groups with 1 variable), and 2-way ANOVA (>1 variable) with appropriate post hoc analyses as indicated in the figure legends. When these parameters were not met, we used the equivalent nonparametric test and post hoc analyses including the Mann-Whitney *U* test and Kruskal-Wallis test with uncorrected Dunn multiple comparisons. *P* values are indicated.

RESULTS

Switching to a Neu5Gc-Free Diet Attenuates Atherogenesis Induced by Dietary Neu5Gc Glycoproteins and Anti-Neu5Gc Antibodies in *Ldlr*$^{-/-}$*Cmah*$^{-/-}$ Mice

In a previous study, we showed that aortic atherosclerosis induced by the HFD feeding of *Ldlr*-deficient mice is aggravated by introducing a human-like *Cmah*-null state via multiple intrinsic mechanisms.[20] This human-like atherosclerosis-prone state was further aggravated by feeding a diet containing the nonhuman Sias Neu5Gc (which is enriched in red meat and metabolically incorporated into human endothelium in vivo)[20] but only if anti-Neu5Gc antibodies were also induced in the same mice, further mimicking the situation of humans who consume processed red meat. To determine whether this xenosialitis phenomenon could be attenuated, we changed the diet of the *Ldlr*$^{-/-}$*Cmah*$^{-/-}$ mice 4 weeks after induction of human-like Neu5Gc antibodies and feeding a Neu5Gc-rich diet, to a Neu5Gc-free diet, identical to the non-Sias soy-based HFD, for a further period of 8 weeks (Gc-HFD→HFD; Figure 1A). This intervention,

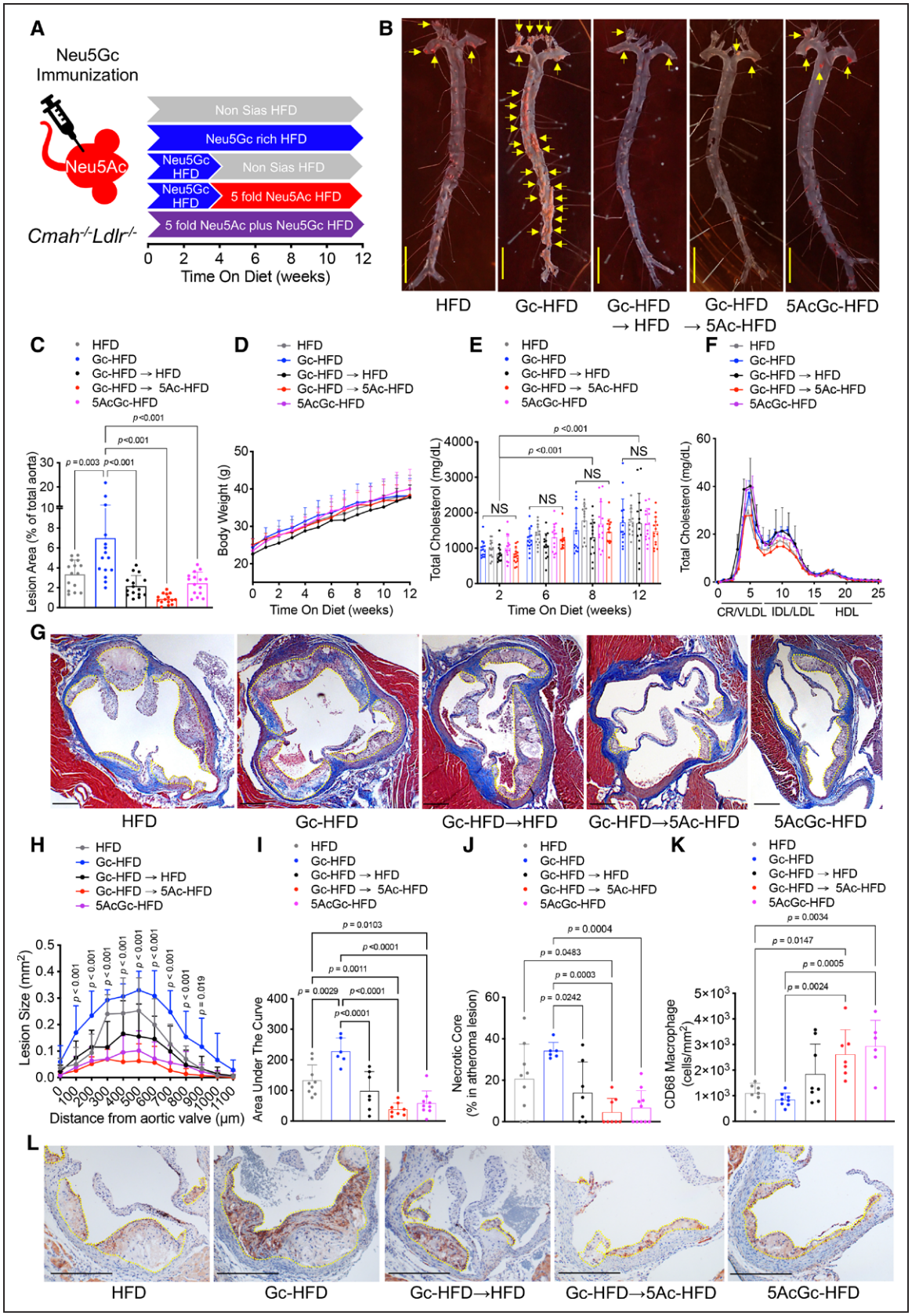

Figure 1. Impact of modulating *N*-glycolylneuraminic acid (Neu5Gc) and *N*-glycolylneuraminic acid (Neu5Ac) dietary content in a human-like xenosialitis atherosclerosis mouse model.

A, *Cmah*$^{-/-}$*Ldlr*$^{-/-}$ mice were immunized with Neu5Gc antigen (Gc immunization) to induce human-like anti-Neu5Gc antibodies, then fed with; non-Sias high-fat diet (HFD) for 12 wk; or Neu5Gc-rich HFD (Gc-HFD) for 12 wk; or Gc-HFD for 4 wk followed by (*Continued*)

analogous to stopping red meat intake in humans, significantly reduced atherosclerotic plaques compared with a continuous Neu5Gc-HFD feeding (Figure 1B and 1C). These differences in sizes of the atherosclerotic plaques were independent of body weight changes (Figure 1D) or plasma cholesterol concentrations (Figure 1E) and despite elevation of atherogenic serum levels of triglyceride-rich lipoproteins (Figure 1F; Figure I in the Data Supplement). Reduction in atherosclerosis plaque size was confirmed by histological analysis using Masson trichrome histochemical stain (Figure 1G; Figure II in the Data Supplement). Importantly, atherosclerotic plaque sizes calculated from serial images taken starting at the aortic valve were significantly smaller, and the size of the necrotic core was reduced, after the diet switch from a Neu5Gc-HFD to non-Sia HFD (Figure 1H through 1J).

Changing From a Neu5Gc-Rich Diet to Neu5Ac-Enriched Diet Caused a Maximum Reduction of Xenosialitis-Mediated Atherosclerotic Plaques

Maximum reduction in atherosclerosis development due to Neu5Gc-mediated xenosialitis was obtained by switching *Ldlr*$^{-/-}$*Cmah*$^{-/-}$ mice, 4 weeks after induction of human-like Neu5Gc antibodies and after feeding a Neu5Gc-rich to a HFD with EBN-derived Neu5Ac (5-fold excess) for another 8 weeks (Gc-HFD→5Ac-HFD; Figure 1A through 1J). The atherosclerotic plaques in the group (Gc-HFD→5Ac-HFD) were significantly smaller and predominantly rich in CD68^{+} macrophages (Figure 1K and 1L). This suggests development of only early fatty streaks and a lack of more intermediate and advanced lesions even when compared with HFD-fed mice. This observation is in stark contrast to the Neu5Gc xenosialitis group (Gc-HFD) where plaques developed substantial necrotic cores, as reported previously[20] (Figure 1J through 1L). This striking protective effect and significant shift to an earlier atherosclerotic plaque morphology is further supported by the almost complete lack of necrotic cores. This observation in the Gc-HFD→5Ac-HFD treatment arm was associated with a reduction in the serum LDL (low-density lipoprotein) cholesterol levels and hepatic triglyceride accumulation (Figure 1F; Figure III in the Data Supplement). Hence, these findings imply that negative cardiovascular effects of Neu5Gc found predominantly in red meat might be counteracted by consuming Neu5Ac-enriched food groups (such as poultry).

Mixing 5-Fold Excess of Neu5Ac-Rich in a Neu5Gc-Rich HFD Reduces Xenosialitis-Mediated Aggravation of Atherosclerosis

We have previously shown in cultured cells that Neu5Ac can metabolically compete for Neu5Gc incorporation.[38] We now asked whether the same type of competition would act in this mouse model of atherosclerosis (Figure 1A). This question was addressed by using the *Ldlr*$^{-/-}$*Cmah*$^{-/-}$ mice, after the induction of human-like Neu5Gc antibodies, and feeding the animals a Neu5Gc-rich HFD but mixed with a 5-fold excess of EBN-derived Neu5Ac, for a period of 12 weeks (5AcGc-HFD). We observed a significant decrease in the size of the atherosclerotic plaques in the 5AcGc-HFD–fed group, compared with the Neu5Gc-HFD–fed group or non-Sias HFD (Figure 1B, 1C, and 1G through 1I). This antiatherogenic effect was observed despite inducing elevated circulating triglyceride-rich lipoproteins (Figure IB in the Data Supplement). Notably, this intervention was also associated with a significant reduction in necrotic core formation and the plaques were macrophage rich, compared with the plaque sizes in animals that did not receive Neu5Gc in their diets although on an HFD (Figure 1J through 1L). These findings suggest that incorporation of Neu5Ac-rich glycoproteins into processed red meat products (or reduced Neu5Gc absorption in general) could result in suppression of Neu5Gc-induced atherogenic events.

Feeding EBN With HFD Has a Strong Protective Effect Against Development of Atherosclerotic Plaques in *Ldlr*$^{-/-}$ Mice but Only in the Human-Like *Cmah*-Null Background

We previously reported that mice with the human-like *Cmah*-null background have an intrinsically increased propensity to developing aortic atherosclerotic plaques when fed an HFD in *Ldlr*-deficient mice.[20] While reanalyzing some of these data, as well as contemporaneous unpublished mouse cohorts, we noted that the propensity

Figure 1 Continued. switching to a non-Sias HFD for 8 wk (Gc-HFD→HFD); or Gc-HFD for 4 wk then switching to 5-fold Neu5Ac-HFD for 8 wk (Gc-HFD→5Ac-HFD); or a premix of 5-fold Neu5Ac over Neu5Gc for 12 wk (5AcGc-HFD; n=14–16, each). **B**, En face analysis of atherosclerotic plaques (yellow arrows), and (**C**) quantification of lipid-rich Sudan IV-positive plaques in aorta. **D**, Body weight change for 12 wk in each group. **E**, Plasma total cholesterol (n=14–16) and (**F**) FPLC analysis of lipoproteins after 12 wk of each HFD feeding (3 pooled plasma from n=4–6 each). **G**, Atherosclerotic plaques in the aortic sinus were evaluated with Masson trichrome (plaques indicated with yellow dotted line and necrotic cores indicated with red dotted lines). **H**, quantification of atherosclerotic plaque size (yellow dots) in the aortic sinus and (**I**) area under the curve, (**J**) necrotic core size (red dots), and (**K**) CD68 infiltration density were calculated in the plaque (yellow dots; n=6–8, each). **L**, Atherosclerotic plaques in the aortic sinus were evaluated with anti-CD68 immunostain. Shown are male data, black bars = 300 μm, mean (SD), 1-Way ANOVA, 2-Way ANOVA with uncorrected Fisher least significant difference post hoc test or Kruskal-Wallis test with uncorrected Dunn multiple comparisons. CR indicates chylomicron remnant; HDL, high-density lipoprotein; IDL, intermediate-density lipoprotein; LDL, low-density lipoprotein; NS, nonsignificant; and VLDL, very-low-density lipoprotein.

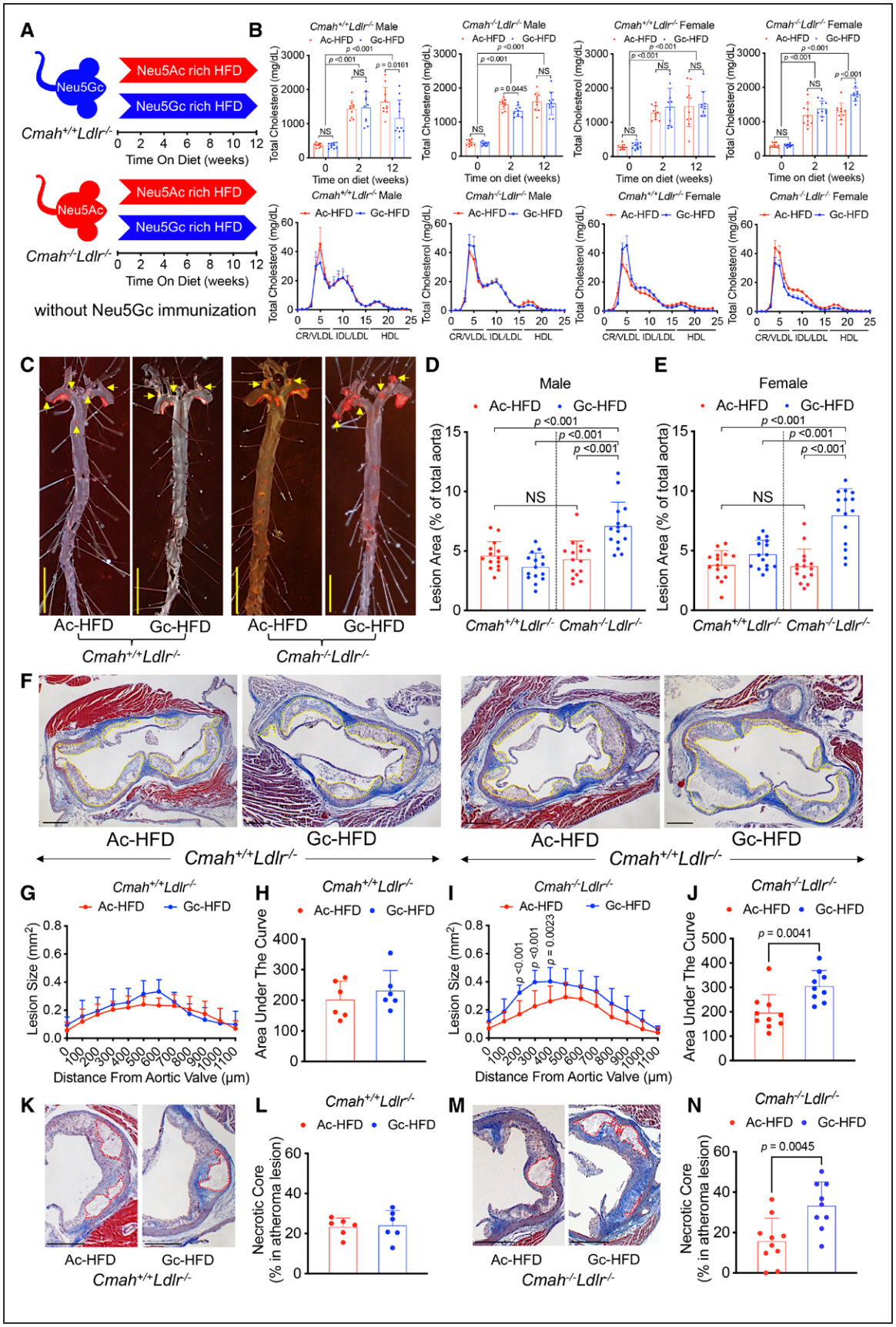

Figure 2. Atheroprotective effect of *N*-glycolylneuraminic acid (Neu5Ac) alone occurs only in human-like *Cmah*$^{-/-}$*Ldlr*$^{-/-}$ mice.
A, Neu5Ac-rich EBN (Ac) or *N*-glycolylneuraminic acid (Neu5Gc)–rich PSM (porcine submaxillary mucin; Gc) were added to a (*Continued*)

to develop atherosclerosis and unstable atheromas is suppressed by feeding of Neu5Ac-rich EBN alone (Figure 2A through 2N). This effect was not seen in the *Ldlr*-deficient *Cmah* wild-type mice (Figure 2A through 2E). These findings indicate that consumption of Neu5Ac-rich EBN can have a major protective effect independent of its impact on red meat and Neu5Gc. Importantly, Neu5Gc-rich HFD itself (without Neu5Gc antibodies) did not change atheromatous plaques when we compare the data with the mice on non-Sia HFD,[20] although some body weight change occurred (Figure IV in the Data Supplement). The Neu5Ac-protective effect was not associated with a reduction in cardiovascular disease risk factors including plasma lipoproteins, such as chylomicron and VLDL (very-low-density lipoprotein) or hepatic lipidosis (Figure 2B; Figures V and VI in the Data Supplement). The Neu5Ac-protective effect did associate with suppression of cytokine expression in macrophages (Figure VII in the Data Supplement). However, no difference in macrophage foam cell conversion was observed with Neu5Ac and Neu5Gc feeding in human THP-1–derived macrophages (Figure VIII in the Data Supplement). Changes in the microbiome induced by adding Neu5Ac or Neu5Gc to the diet are also potential contributing factors.[30]

DISCUSSION

This work brings together several seemingly disparate fields: EBN as a traditional Chinese health food, also known as Collocalia mucoid, long recognized by Sias researchers as a rich source of the human dominant sialic Neu5Ac; a novel mechanism for the risk of increased atherosclerosis associated with red meat consumption; and potential interventions using Collocalia mucoid—the primary component of Sia-rich EBN. There are some prior studies indicating that EBN could ameliorate HFD-induced hyperlipidemia and hypercoagulation[39] and insulin resistance,[40,41] but these studies were done in rats (which unlike our mouse model did not have the human-like *Cmah* deficiency). The mechanisms involved are unclear, and it was suggested that EBN might reduce oxidative stress via upregulation of hepatic antioxidant genes and contribute to the downregulation of inflammatory cytokine genes such as *CCL2* or *IL-6*.[39] We did also observe a reduction in cytokine expression in isolated peritoneal macrophage from EBN-fed mice, suggesting attenuated systemic inflammation (Figure VII in the Data Supplement). Further studies, however, need to determine to what extent this anti-inflammatory effect is a direct or indirect result of the EBN feeding. There have also been reports that feeding high levels of free Neu5Ac by gavage can attenuate HFD-induced hyperlipidemia and associated hypercoagulation[42] and insulin resistance,[43] again in a rat model. Free Neu5Ac administration to HFD-fed *ApoE*$^{-/-}$ mice also decreased aortic atherosclerotic plaque formation by 18.9% and decreased the lipid deposition in liver hepatocytes by 26.7%. Also noted were a 62.6% reduction of triglyceride by improving lipoprotein lipase activity, 17.5% reduction of the plasma total cholesterol by upregulating reverse cholesterol transport–related protein expression such as ABC (ATP-binding cassette transporter)-G1 and ABCG5 in liver or small intestine, and reducing oxidative stress by increasing antioxidant enzymes activity and protein expression of paraoxonase 1.[44]

However, none of the above studies were done in a human-like CMAH-null background. Also, the in vivo kinetics of orally administered free Sias (rapid excretion in the urine)[45–47] is markedly different from that of the glycosidically bound Neu5Ac in EBN, and thus any mechanism of action is likely different. Additionally, feeding such high levels of free Neu5Ac could potentially cause dysbiosis involving microorganisms found in the gut lumen, which utilize Neu5Ac for their metabolism and growth.[48] What remains notable is our observation that intervention with EBN Neu5Ac after a short-term Neu5Gc regime almost completely attenuates atherosclerosis formation. This effect is especially remarkable as *Ldlr*-deficient mice on a high-fat and high-cholesterol diet for 12 weeks almost always robustly develop advanced atherosclerotic lesions. The observation underscores the therapeutic potential of dietary Neu5Ac development in treatment and possibly also regression of atherosclerosis in at-risk cardiovascular disease patients.

Recent work has highlighted that inhibition of the sialidase, Neu1 (neuraminidase 1), is associated with reduced atherogenesis in mice.[49] Potentially one can envision that elevated circulating Neu5Ac levels due to feeding can attenuate sialidase activity and hence reduce atherogenesis in our model. Such inhibition would also prevent the formation of atherogenic desialylated LDL.[50] However, free Neu5Ac is primarily found in the β-anomeric form and sialidases act on the α-anomer bound to glycans. Thus,

Figure 2 Continued. soy-based non-Sias high-fat diet (HFD; Ac-HFD or Gc-HFD), feeding *Cmah*$^{-/-}$*Ldlr*$^{-/-}$ and *Cmah*$^{+/+}$*Ldlr*$^{-/-}$ mice during 6 to 18 wk, without any prior immunization. **B**, Plasma total cholesterol (n=14–16) and FPLC analysis of lipoproteins after 12 wk of each HFD feeding (3 pooled serums from male and female mice, n=4–6) as Figure 1. **C**, En face analysis of atherosclerosis (red dots and yellow arrow show in female atheroma lesions, yellow bar=500 μm), and quantification of Sudan IV-positive area I shown in male (**D**), and female (**E**; n=14–16). **F**, *Cmah*$^{+/+}$*Ldlr*$^{-/-}$ and *Cmah*$^{-/-}$*Ldlr*$^{-/-}$ female were analyzed 12 wk after Neu5Ac or Neu5Gc added HFD feeding for atherosclerotic plaque development in the aortic root (n=6 each). **G**–**J**, Quantification of total atherosclerotic plaque size (white dotted lines) in the aortic sinus with Masson trichrome stain; area under the curve data shown. **K**–**N**, Necrotic core (red dotted lines) size analysis. Shown are black bars, 300 μm, mean (SD), unpaired 2-tailed Student *t* test, Mann-Whitney *U* test, 1-way ANOVA, 2-way ANOVA, with uncorrected Fisher least significant difference post hoc test or Kruskal-Wallis test with uncorrected Dunn multiple comparisons. CR indicates chylomicron remnant; HDL, high-density lipoprotein; IDL, intermediate-density lipoprotein; LDL, low-density lipoprotein; NS, nonsignificant; and VLDL, very-low-density lipoprotein.

increased free Neu5Ac in our studies should not inhibit Neu1 activity. In addition, Neu5Ac glycoproteins levels are abundant in the serum (≈2 mmol/L) compared with the small amounts of dietary Neu5Ac glycoproteins from typical human diets (0.8–4 μmol Neu5Ac per gram diet). Moreover, Neu1 has no substrate preference between α2-3–linked and α2-6–linked Neu5Ac or Neu5Gc—the main linkages found on N-glycosylated proteins.[51] RNA sequencing analysis of *Cmah*$^{-/-}$ macrophage also did not report any differential *Neu1* expression, even in the context of coincubating macrophages with 2 mmol/L Neu5Ac or Neu5Gc suggesting no preferential regulation or inhibition of Neu1 by either Neu5Ac or Neu5Gc.[52] Nevertheless, future studies will have to assess the importance of Neu1 activity in the observed Neu5Ac antiatherogenic effect in humanized CMAH-deficient mice.

Weight for weight, EBN may now be among the most expensive animal products consumed by humans. This traditional Chinese health food has been claimed to have many medical benefits and is recommended as a tonic for elderly people, pregnant women, and growing children. However, none of these claims have been corroborated by controlled and blinded clinical studies. It is also necessary to validate the quality of the product because analysis of EBN showed some possible allergens, such as mites, and microorganisms, contaminated especially in raw EBN.[53,54] Meanwhile, in addition to the increasing encroachment of humans on the birds' habitat, pollution is now eroding some of the caves where they live and build their nests. Rising prices are also leading the harvesters of nests to become more aggressive, sometimes snatching nests as soon as they are built or grabbing nests that have eggs in them.[31,55] With increasing demand, there are also now reinforced concrete nesting houses established in urban areas near the sea, since the birds have a propensity to flock in such places.

If the claimed health benefits of EBN are indeed primarily due to the high content of Neu5Ac,[56] there are other far less expensive alternatives generated as byproducts of the poultry and dairy industry[57–59] that should serve as suitable substitutes for EBN in general. Thus, one can suggest production of an imitation EBN, which could have much of the claimed health benefits of EBN without the high cost or the negative affect on the ecologically pressured bird species. These or other less expensive Neu5Ac-rich EBN substitutes may perhaps even help to protect these species from endangerment.

Finally, while doing these studies, we noticed also that the simultaneous addition of a 5-fold excess of glycoprotein-bound Neu5Ac substantially blunted the xenosialitis effect of Neu5Gc. This is likely because the two molecules derived from the same meal are simultaneously entering cells at the same time and are in competition. This finding suggests that foods containing a substantial amount of Neu5Ac in great excess over Neu5Gc may not be associated with disease risk. This ratio may perhaps explain why most milk products are not associated with cancer risk. Of course, given the variation in expression of CMAH even within species and between tissue types, each food source must be directly assayed for this ratio.

ARTICLE INFORMATION

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Disclosures

None.

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SUPPLEMENTAL MATERIALS

Dietary Neu5Ac Intervention Protects Against Atherosclerosis Associated with Human-Like Neu5Gc loss

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Key words: Human Evolution, Species Conservation, Edible Bird's nest, Sialic acid, Atherosclerosis, *N*-acetylneuraminic acid (Neu5Ac), *N*-glycolylneuraminic acid (Neu5Gc), Cytidine monophosphate (CMP)-*N*-acetylneuraminic acid hydroxylase (CMAH).

Running title: Dietary Neu5Ac protects against atherosclerosis

This section includes:

Online Figures I to VIII
Online Tables I and II

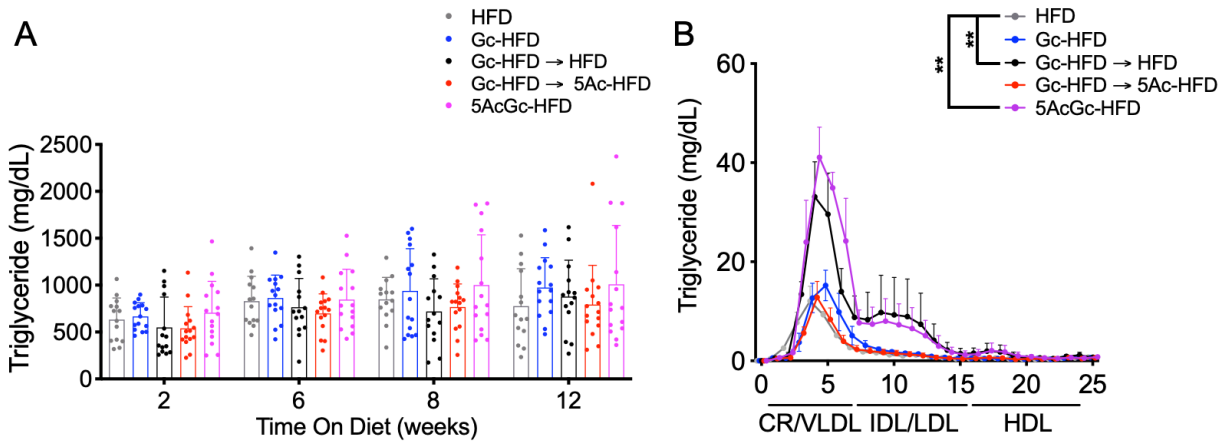

Figure I. Plasma triglyceride analysis in a human-like "xenosialitis" model.

Male *Cmah*$^{-/-}$*Ldlr*$^{-/-}$ mice that were immunized with Neu5Gc antigen to induce anti-Neu5Gc antibodies, then fed with non-Sias high fat diet (HFD) for 12 weeks, Neu5Gc rich high fat diet (Gc-HFD) for 12 weeks, Gc-HFD (4 weeks) then switched to HFD (8 weeks) (Gc-HFD→HFD), Gc-HFD for 4 weeks, then switched to 5-fold Neu5Ac HFD (Gc-HFD→5Ac-HFD) for 8 weeks, or premix of 5-fold Neu5Ac and Neu5Gc contained HFD (5AcGc-HFD) for 12 weeks (male, n = 14 - 16 each). **(A)** Plasma triglyceride analysis, and **(B)** FPLC for pooled sera (3 pooled samples from n = 3–5 each)**.** Mean (SD), Two-way ANOVA or Kruskal-Wallis test; uncorrected Fisher LSD post hoc test or uncorrected Dunn's multiple comparisons post hoc test were conducted,$^{**}p < 0.01$.

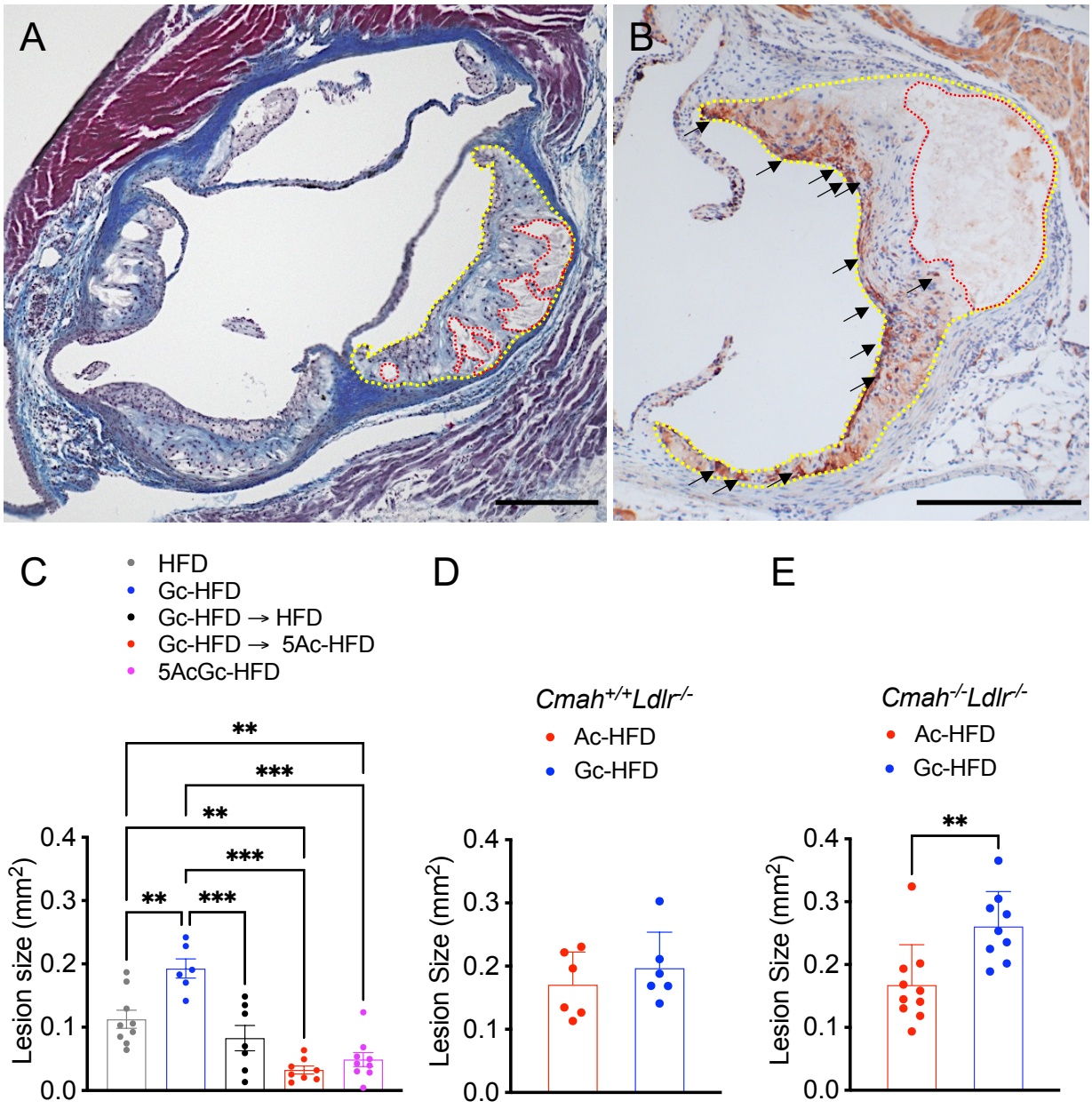

Figure II. Histological analyses of atheroma lesion.

(A) Necrotic core (red dots) without any cell components were developed in inside of the atheroma lesion (yellow dots) in a Masson's trichrome section. **(B)** CD68 positive macrophage infiltration (black arrow) was detected in the upper side of the lesion, and the necrotic core was formed in the deeper lesion (red dots). Black bars showed 300 μM. **(C-E)** Lesion size of the atheroma was calculated by mean of each segment from in main

Fig.1H and Fig.2G and I, Mean (SD), Student t-test and One-way ANOVA, $^{**}p < 0.01$, $^{***}p < 0.001$.

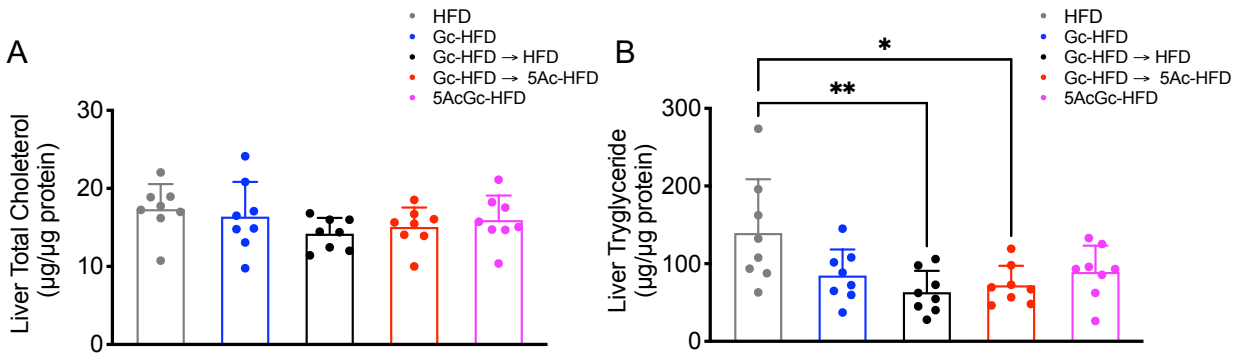

Figure III. Hepatic lipid content in the human-like "xenosialitis" models.

Male *Cmah*$^{-/-}$*Ldlr*$^{-/-}$ mice that were divided into 5 groups as described in Fig. S1. **(A)** Total cholesterol and **(B)** triglyceride of homogenized liver were measured after 12 weeks feeding (n = 8 each)**.** Mean (SD), One-way ANOVA, *$p < 0.05$, **$p < 0.01$.

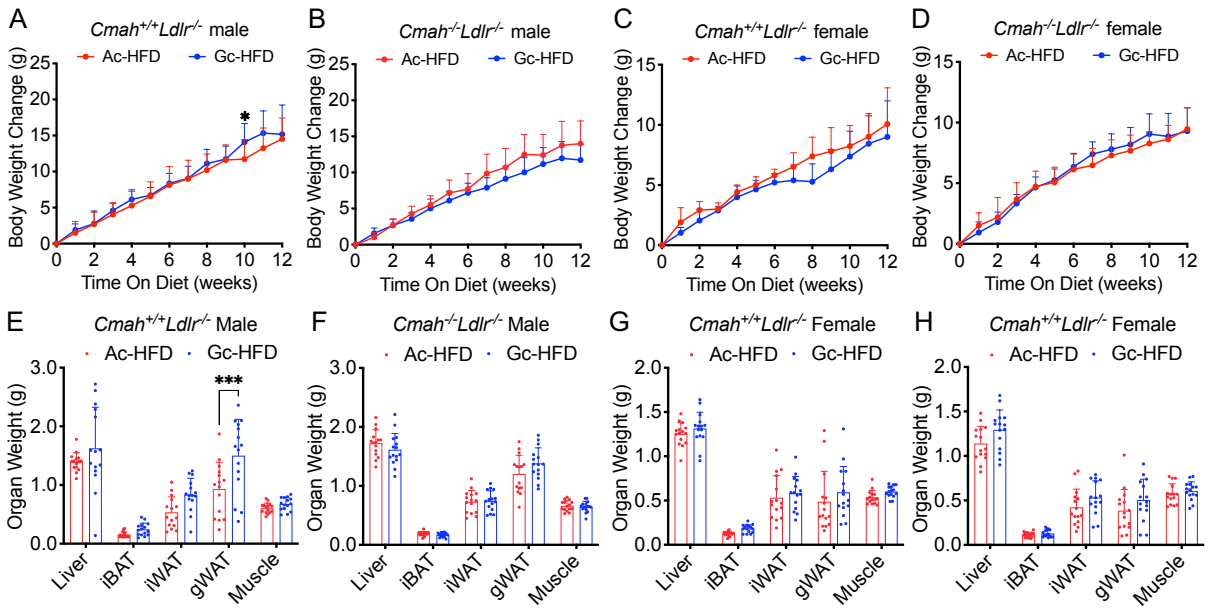

Figure IV. Total body weight and organ weight in *Cmah*$^{-/-}$*Ldlr*$^{-/-}$ and *Cmah*$^{+/+}$*Ldlr*$^{-/-}$ fed with Neu5Ac rich or Neu5Gc rich high fat diet.

Six weeks old *Cmah*$^{+/+}$*Ldlr*$^{-/-}$ and *Cmah*$^{-/-}$*Ldlr*$^{-/-}$ male and female mice were fed with Neu5Ac (Ac) or Neu5Gc (Gc) added soy based high fat diet (HFD) for 12 weeks. **(A) - (D)** Body weight changes (male and female, n = 14 - 16). **(E) - (H)** Organs weight at 12 weeks: liver; interscapular brown adipose tissue, iBAT; inguinal subcutaneous white adipose tissue, iWAT; gonadal white adipose tissue, gWAT; biceps femoris muscle (male and female, n = 14 - 16). Mean (SD), Two-way ANOVA or Kruskal-Wallis test; uncorrected Fisher LSD post hoc test or uncorrected Dunn's multiple comparisons post hoc test were conducted, *$p < 0.05$, ***$p < 0.001$.

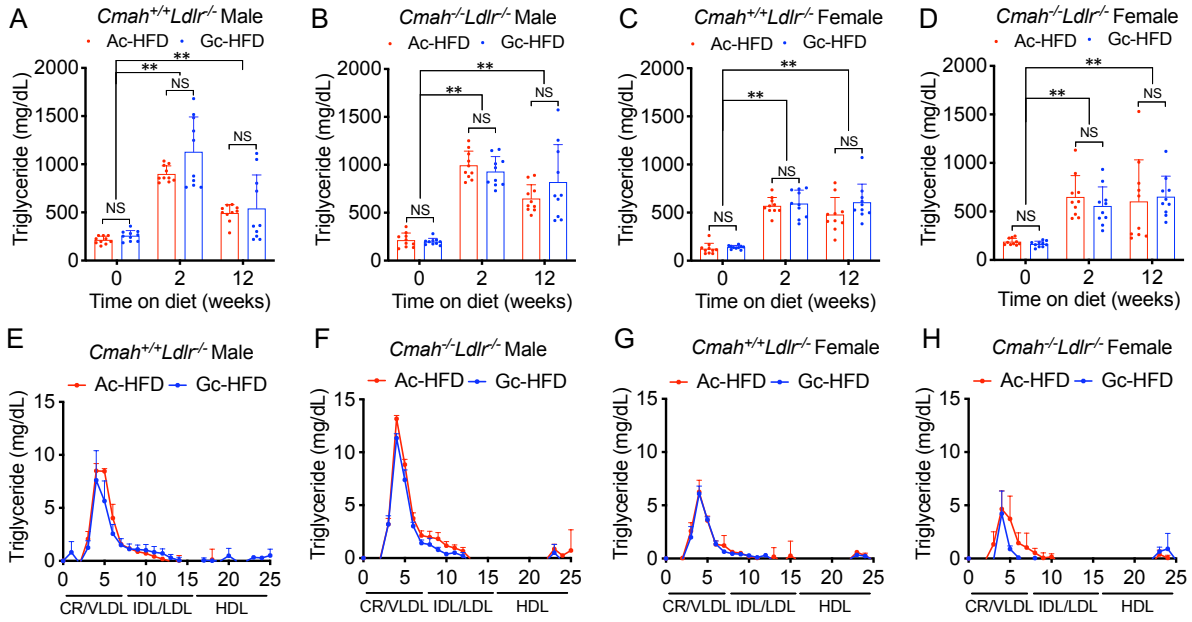

Figure V. Triglyceride analysis in *Cmah*$^{+/+}$*Ldlr*$^{-/-}$ and *Cmah*$^{-/-}$*Ldlr*$^{-/-}$ mice.

Six weeks old *Cmah*$^{+/+}$*Ldlr*$^{-/-}$ and *Cmah*$^{-/-}$*Ldlr*$^{-/-}$ male and female mice were fed with Neu5Ac (Ac) or Neu5Gc (Gc) added soy based HFD for 12 weeks. **(A)** - **(D)** Plasma total triglyceride, and **(E) - (H)** FPLC analysis 12 weeks after Ac-HFD or Gc-HFD feeding (male and female, n = 4-6 each). Mean (SD), Two-way ANOVA or Kruskal-Wallis test; uncorrected Fisher LSD post hoc test or uncorrected Dunn's multiple comparisons post hoc test were conducted, $^{**}p < 0.01$.

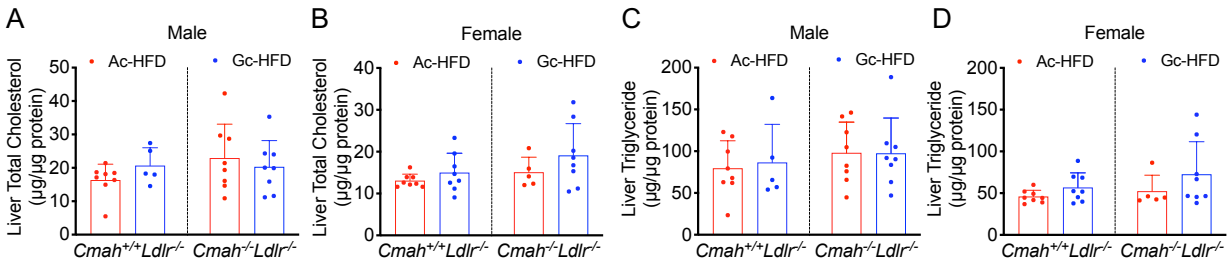

Figure VI. Hepatic lipidosis in *Cmah*$^{+/+}$*Ldlr*$^{-/-}$ and *Cmah*$^{-/-}$*Ldlr*$^{-/-}$ mice.

Six weeks old *Cmah*$^{+/+}$*Ldlr*$^{-/-}$ and *Cmah*$^{-/-}$*Ldlr*$^{-/-}$ male and female mice were fed with Neu5Ac (Ac) or Neu5Gc (Gc) added soy based HFD for 12 weeks. **(A)** - **(D)** Plasma total triglyceride, and **(E) - (H)** FPLC analysis during Ac-HFD or Gc-HFD feeding (male and female, n = 5-8 each). **(I) - (L)** Mean (SD), Two-way ANOVA; no significance detected.

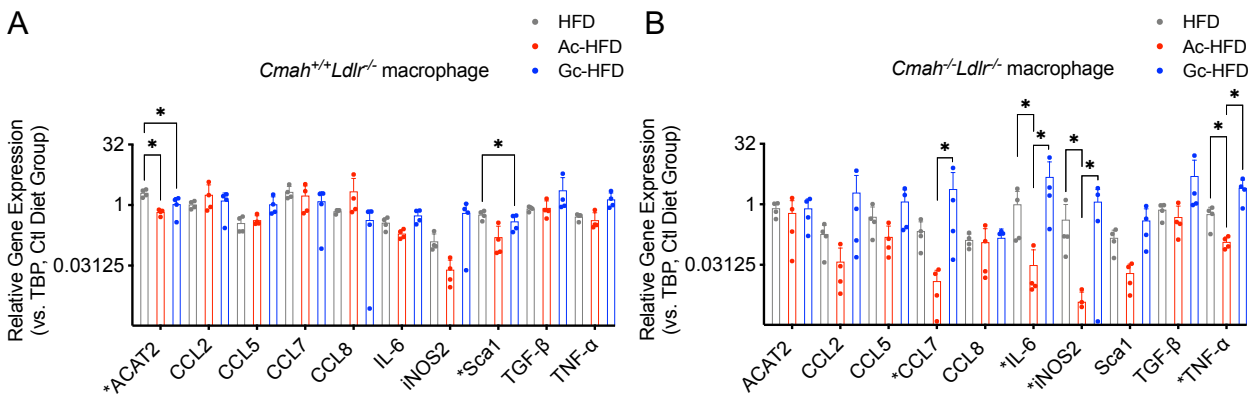

Figure VII. Neu5Ac downregulate macrophages cytokine expression in Human-like *Cmah*$^{-/-}$*Ldlr*$^{-/-}$

Cytokine gene expression in peritoneal macrophages collected from **(A)** *Cmah*$^{+/+}$*Ldlr*$^{-/-}$ or **(B)** *Cmah*$^{-/-}$*Ldlr*$^{-/-}$ female fed with sialic acid (sias) free soy based control diet (Ctl Diet), non-sias HFD, Neu5Ac rich HFD (Ac-HFD), or Neu5Gc rich HFD (Gc-HFD) for 12 weeks (n = 4 each). Expressions of inflammatory cytokines were normalized with TBP and Ctl Diet group. Mean (SD), One-way ANOVA, *$p < 0.05$.

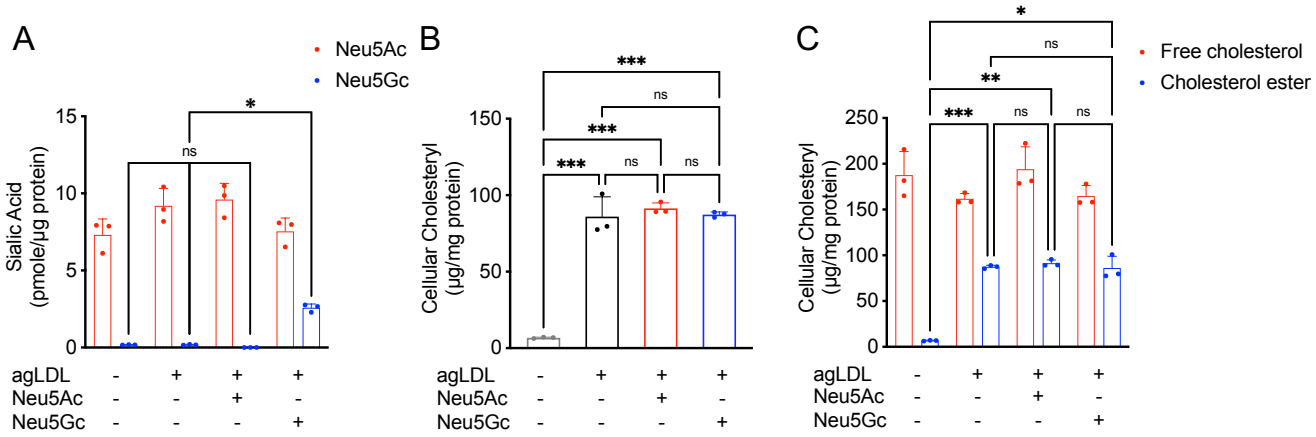

Figure VIII. Sialic acids feeding did not change cholesterol uptake in human macrophage-like cells

Human macrophage-like cells (THP-1) were fed with aggregated LDL for foam cell formation. **(A)** Sialic acid of THP-1 cell lysate were measured by HPLC after 24 hrs feeding with free Neu5Ac or Neu5Gc. **(B)**Total cholesterol levels and **(C)** Free cholesterol and cholesterol ester levels were analyzed by ELISA. Mean (SD), One-way ANOVA and Two-way ANOVA with uncorrected Fisher LSD post hoc test,*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

Table I. Contents of various chows used in this study

Chow type	Source	Contains Soy	Contains Neu5Ac	Contains Neu5Gc	Other descriptions
HFD	Soy	+	-	-	Ctrl, Sia-free, Soy HFD
Gc-HFD	Porcine Submaxillary gland mucin	+	Traces	+	PSM, red-meat mimicking, Gc-HFD
Ac-HFD	Edible bird's nest (Collocolia mucoid)	+	+	Traces	EBN, Ac-HFD
5AcGc-HFD	Mixture of PSM and EBN	+	+	+	Premixed chow

All chows are in a Soy high-fat diet base that does not contain sialic acids. Sources mentioned above are additional additions to this base diet to introduce sialic acids (Neu5Ac and / or Neu5Gc). 5AcGc-HFD contains 5x Neu5Ac for every 1x Neu5Gc.

Table II. qPCR Primers

Gene	Forward primer (5'-3')	Reverse primer (5'-3')
Acat2	GACTTGGTGCAATGGACTCG	GGTCTTGCTTGTAGAATCTGG
Ccl2 (Mcp--1)	AGGTCCCTGTCATGCTTCTG	GCTGCTGGTGATCCTCTTGT
Ccl5 (Rantes)	CATATGGCTCGGACACCA	ACACACTTGGCGGTTCCT
Ccl7 (Mcp-3)	CCTGGGAAGCTGTTATCTTCAA	TGGAGTTGGGGTTTTCATGTC
Ccl8 (Mcp-2)	GCTGTGGTTTTCCAGACCAA	GAAGGTTCAAGGCTGCAGAA
Il-6	CCAGAGATACAAAGAAATGATGG	ACTCCAGAAGACCAGAGGAAAT
iNos2	GTTCTCAGCCCAACAATACAAGA	GTGGACGGGTCGATGTCAC
Sca1 (Ly6A)	ATGGACACTTCTCACACTACAAAG	TCAGAGCAAGGTCTGCAGGAGGACTG
Tbp	GAAGCTGCGGTACAATTCCAG	CCCCTTGTACCCTTCACCAAT
TgFβ	GGAGAGCCCTGGATACCAAC	AAGTTGGCATGGTAGCCCTT
Tnfα	CCAGACCCTCACACTCAGATC	CACTTGGTGGTTTGCTACGAC

Major Resources Table

In order to allow validation and replication of experiments, all essential research materials listed in the Methods should be included in the Major Resources Table below. Authors are encouraged to use public repositories for protocols, data, code, and other materials and provide persistent identifiers and/or links to repositories when available. Authors may add or delete rows as needed.

Animals (in vivo studies)

Species	Vendor or Source	Background Strain	Sex	Persistent ID / URL
Cmah$^{-/-}$ *Ldlr*$^{-/-}$ Mus musculus	UCSD	C57BL/6N	M/F	IMSR Cat# JAX:017588, RRID:IMSR_JAX:017588
Cmah$^{+/+}$ *Ldlr*$^{-/-}$ Mus musculus	UCSD	C57BL/6N	M/F	IMSR Cat# MUGEN:M191001, RRID:IMSR_MUGEN:M191001

Genetically Modified Animals

	Species	Vendor or Source	Background Strain	Other Information	Persistent ID / URL
Parent-Male	*Cmah*$^{-/-}$ Mus musculus	UCSD	C57BL/6N	*Mol Cell Biol* **27**, 4340 (2007)	N/A
Parent - Female	*Ldlr*$^{-/-}$ Mus musculus	UCSD	C57BL/6N	*J Clin Invest* **92**, 883 (1993)	N/A

Antibodies

Target antigen	Vendor or Source	Catalog #	Working concentration	Lot # (preferred but not required)	Persistent ID / URL
Mouse CD68	Abcam	ab125212	1:50 dilution	AB_10975465	N/A

DNA/cDNA Clones

Clone Name	Sequence forward primer (5'-3')	Sequence reverse primer (5'-3')	Persistent ID / URL
Acat2	GACTTGGTGCAATGGACTCG	GGTCTTGCTTGTAGAATCTGG	N/A
Ccl2 (Mcp--1)	AGGTCCCTGTCATGCTTCTG	GCTGCTGGTGATCCTCTTGT	N/A
Ccl5 (Rantes)	CATATGGCTCGGACACCA	ACACACTTGGCGGTTCCT	N/A
Ccl7 (Mcp-3)	CCTGGGAAGCTGTTATCTTCAA	TGGAGTTGGGGTTTTCATGTC	N/A
Ccl8 (Mcp-2)	GCTGTGGTTTTCCAGACCAA	GAAGGTTCAAGGCTGCAGAA	N/A
Il-6	CCAGAGATACAAAGAAATGATGG	ACTCCAGAAGACCAGAGGAAAT	N/A
iNos2	GTTCTCAGCCCAACAATACAAGA	GTGGACGGGTCGATGTCAC	N/A
Sca1 (Ly6A)	ATGGACACTTCTCACACTACAAAG	TCAGAGCAAGGTCTGCAGGAGGACTG	N/A
Tbp	GAAGCTGCGGTACAATTCCAG	CCCCTTGTACCCTTCACCAAT	N/A
TgFβ	GGAGAGCCCTGGATACCAAC	AAGTTGGCATGGTAGCCCTT	N/A
Tnfα	CCAGACCCTCACACTCAGATC	CACTTGGTGGTTTGCTACGAC	N/A

Cultured Cells

Name	Vendor or Source	Sex (F, M, or unknown)	Persistent ID / URL
Peritoneal macrophage	*Cmah*$^{-/-}$ *Ldlr*$^{-/-}$ or *Cmah*$^{+/+}$ *Ldlr*$^{-/-}$	F	N/A

Data & Code Availability

Description	Source / Repository	Persistent ID / URL

Other

Description	Source / Repository	Persistent ID / URL

DOI [to be added]

Chimpanzee red blood cell plasma membranes	Chimpanzee undergoing routine health checks at Yerkes National Primate Research Center	N/A (Emory University IACUC approved)
Freund's Adjuvant, Complete cell suspension	Sigma-Aldrich	Cat.No. F5881
Freund's Adjuvant, Incomplete liquid	Sigma-Aldrich	Cat.No. F5506
Pig Submaxillary Glands	Pel-Freez Arkansas LLC	N/A
White Bird's Nest AAA	Golden Nest	N/A
N-Acetylneuraminic Acid (Neu5Ac, NANA)	Nacalai	Cat.No. 08371
N-Glycolylneuraminic Acid (Neu5Gc, NGNA)	Inalco	Cat.No.1758-9850
CHOLESTEROL, TOTAL-SL	SEKISUI DIAGNOSTIC	Cat.No. 234-60
TRIGLYCERIDE-SL	SEKISUI DIAGNOSTIC	Cat.No. 236-60
ImageJ	https://imagej.nih.gov/ij/	N/A
Prism 9	GraphPad	N/A

DOI [to be added]