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Murine Norovirus Increases Atherosclerotic Lesion Size and Macrophages in *Ldlr*$^{-/-}$ Mice

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Murine norovirus (MNV) is prevalent in rodent facilities in the United States. Because MNV has a tropism for macrophages and dendritic cells, we hypothesized that it may alter phenotypes of murine models of inflammatory diseases, such as obesity and atherosclerosis. We examined whether MNV infection influences phenotypes associated with diet-induced obesity and atherosclerosis by using *Ldlr*$^{-/-}$ mice. Male *Ldlr*$^{-/-}$ mice were maintained on either a diabetogenic or high-fat diet for 16 wk, inoculated with either MNV or vehicle, and monitored for changes in body weight, blood glucose, glucose tolerance, and insulin sensitivity. Influence of MNV on atherosclerosis was analyzed by determining aortic sinus lesion area. Under both dietary regimens, MNV-infected and control mice gained similar amounts of weight and developed similar degrees of insulin resistance. However, MNV infection was associated with significant increases in aortic sinus lesion area and macrophage content in *Ldlr*$^{-/-}$ mice fed a high-fat diet but not those fed a diabetogenic diet. In conclusion, MNV infection exacerbates atherosclerosis in *Ldlr*$^{-/-}$ mice fed a high-fat diet but does not influence obesity- and diabetes-related phenotypes. Increased lesion size was associated with increased macrophages, suggesting that MNV may influence macrophage activation or accumulation in the lesion area.

Abbreviations: HFHS, high-fat high-sucrose; IPGTT, intraperitoneal glucose tolerance test; IPITT, intraperitoneal insulin tolerance test; Ldlr, low-density lipoprotein receptor; MNV, murine norovirus.

Murine norovirus (MNV) is a single-strand RNA virus and belongs to the *Caliciviridae* family.[5] MNV is widespread in SPF facilities in the United States, Canada, Asia, and Europe.[10,11,16,21,25,32] MNV does not cause any overt disease in mice with a competent immune system but causes serious illness in mice with specific, genetically modified immunodeficiencies.[14,27] We found that MNV infection can accelerate the progression of inflammatory bowel disease in *Mdr1a*$^{-/-}$ mice, a strain that is immunocompetent yet genetically susceptible to the disease.[18] In addition, interactions between dendritic and T cells were altered in MNV-infected mice.[18] These findings raised the possibility that MNV, with its proclivity to infect dendritic cells and macrophages,[29] might influence disease progression in murine models of other inflammatory diseases, such as obesity and atherosclerosis. We first tested this possibility by examining whether MNV infection alters phenotypes associated with obesity and diabetes in C57BL/6 mice fed a high-fat diet (60% calories from fat).[24] We found that MNV infection did not influence obesity- or diabetes-related phenotypes such as rate of weight gain, fasting blood glucose, glucose tolerance, and insulin sensitivity. However, MNV-infected mice had subtle alterations in lymphoid tissue, including increased reactive hyperplasia of mesenteric lymph nodes.[24] These data raised concerns that, given that MNV is endemic and prevalent in mouse colonies, MNV infection could be a potential confounding variable in murine studies of other inflammatory diseases, such as atherosclerosis.[9]

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Mice are naturally resistant to atherosclerosis and require genetic manipulation to increase their susceptibility. One of the most commonly used mouse models of atherosclerosis is *Ldlr*$^{-/-}$ mice. These mice become dyslipidemic due to impaired lipoprotein metabolism, resulting in the susceptibility to atherosclerosis.[2,13] As a consequence, *Ldlr*$^{-/-}$ mice develop pronounced atherosclerosis when fed an atherogenic diet (high fat, high cholesterol). Here we report our studies to examine whether MNV alters the development or progression of atherosclerosis in *Ldlr*$^{-/-}$ mice. In addition, we examined whether MNV infection changes obesity- and diabetes- related phenotypes in these mice to confirm our previous findings in C57BL/6 mice.[24]

Materials and Methods

Animals. Male B6.129S7-*Ldlr*^{tm1Her}/J (*Ldlr*$^{-/-}$) mice (age, 5 wk) were purchased from Jackson Laboratory (Bar Harbor, ME) and acclimated in an SPF facility in a temperature-controlled room (20 to 23 °C) with a 12:12-h light:dark cycle. Infectious agents excluded from our facility include mouse hepatitis virus, mouse parvovirus, minute virus of mice, reovirus 3, pneumonia virus of mice, epizootic diarrhea of infant mice, Theiler murine encephalomyelitis virus, lymphocytic choriomeningitis virus, ectromelia virus, Sendai virus, sialodacryoadenitis virus, rat parvoviruses, *Mycoplasma pulmonis*, pinworms, and fur mites. In addition, *Helicobacter* spp. and MNV were excluded in our room except for experimental infection. At the end of the study, *Helicobacter* spp. status was determined as negative from fecal PCR as established in our laboratory.[3] *Ldlr*$^{-/-}$ mice were tested by the supplier to be free of MNV; we confirmed this status by PCR testing of feces before the start of the diet study. Standard operating protocols

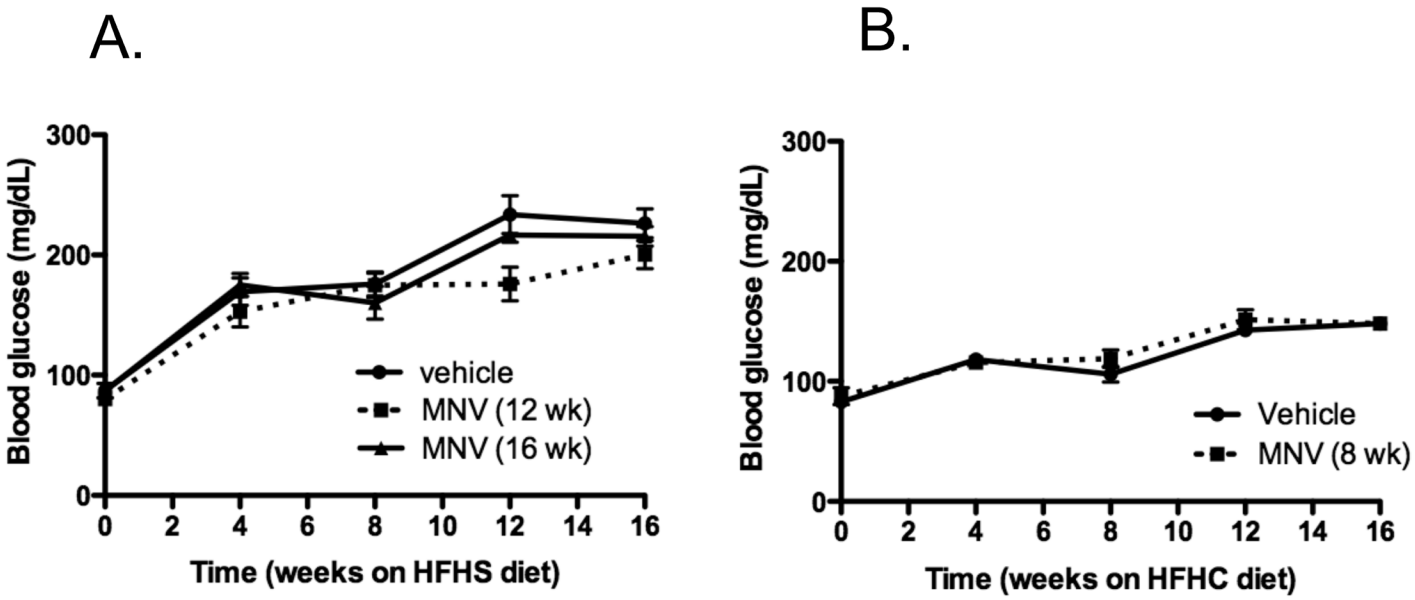


Figure 1. Fasting blood glucose increased with time in *Ldlr*$^{-/-}$ mice fed either a HFHS diet or a HFHC diet. Blood glucose was measured every 4 wk from tail blood following overnight fasting. (A) Fasting blood glucose in study 1 (HFHS diet). (B) Fasting blood glucose in study 2 (HFHC diet). No differences were observed among mice infected and uninfected with MNV at each time point (one-way ANOVA or *t* test).

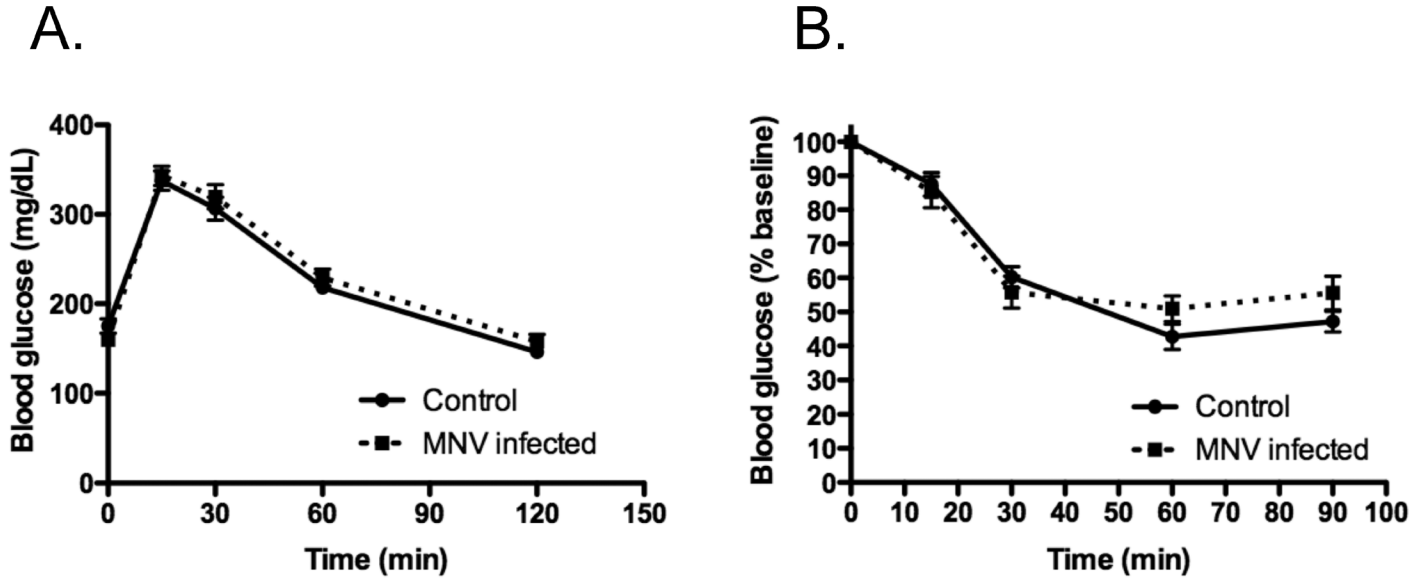


Figure 2. (A) IPGTT and (B) IPITT were performed after overnight fasting at 11 and 12 wk after diet initiation (HFHC diet). MNV infection occurred at 8 wk after diet initiation and did not significantly influence glucose tolerance or insulin sensitivity in these mice (2-way ANOVA).

used in our mouse housing were designed to keep MNV infection only in intended treatment groups; these practices included using 2 separate changing stations for infected and noninfected animals.[24] All experimental procedures involving animals were reviewed and approved by the University of Washington IACUC.

Diets and MNV infection. Mice were acclimated for a week before being assigned to treatment groups. During the acclimation period, mice were given rodent chow (PicoLab Rodent Diet 20, 5053, LabDiet, St Louis, MO). For study 1, all mice received high-fat, high-sucrose diet (HFHS; S1850 Diabetogenic Diet, BioServ, Frenchtown, NJ) for 16 wk to induce obesity and diabetes. Mice were assigned to 1 of 3 groups (n = 12 mice per group): vehicle treatment (control), MNV infection at the start of the diet (MNV, 16 wk), and MNV infection 4 wk after the start of the diet (MNV, 12 wk). For study 2, all mice were given a Western-type diet (high-fat, high-cholesterol (HFHC) diet; TD88137, Harlan Teklad, Madison, WI) for 16 wk to induce atherosclerosis. Mice were assigned to 1 of 2 treatment groups (n = 18 mice per group): vehicle treatment (control) and MNV infection at 8 wk after the start of the diet. For both studies, mice were weighed weekly, and 7-d food intakes were determined at 4 wk after the initiation of the diet and immediately after MNV infection.

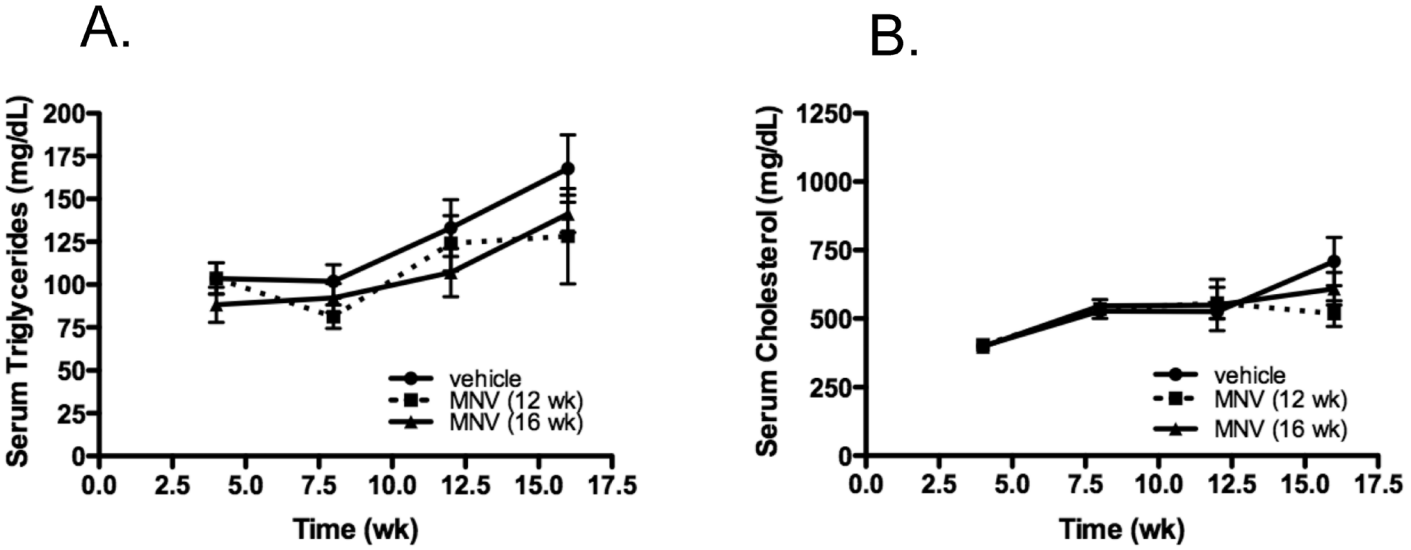


Figure 3. Serum lipids after overnight fasting were determined throughout the feeding of a HFHS diet. Both (A) triglyceride and (B) cholesterol levels increase during the diet period regardless of MNV infection status. No significant differences were found among treatment groups at each time point (one-way ANOVA).

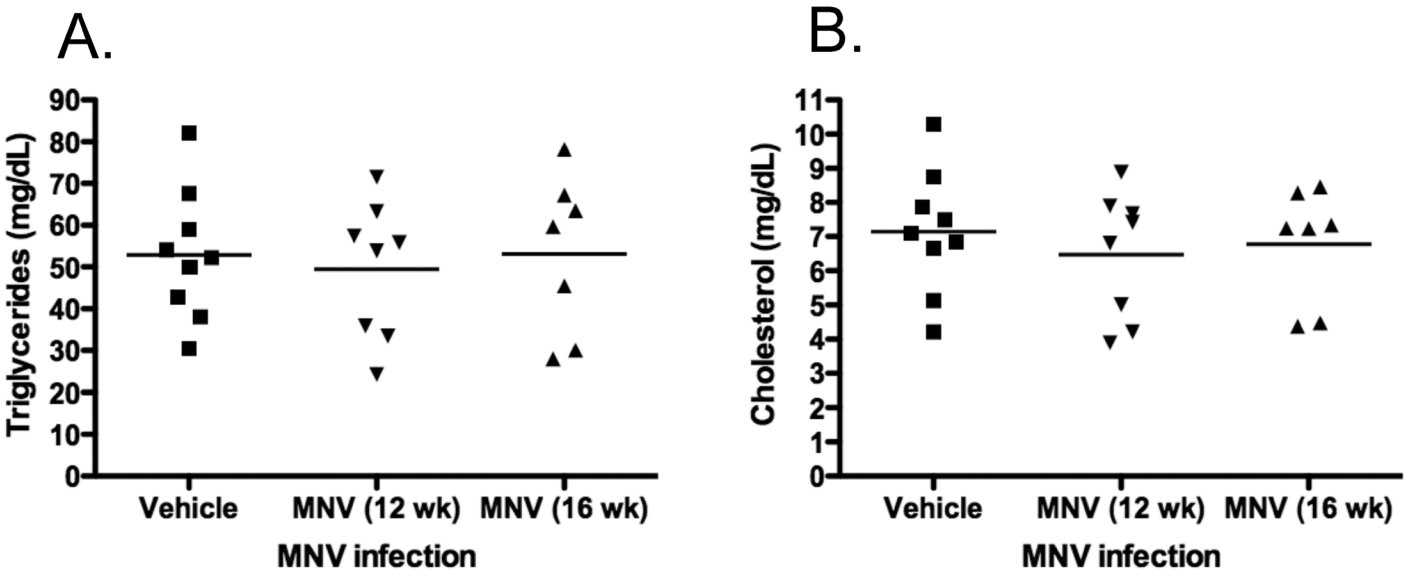
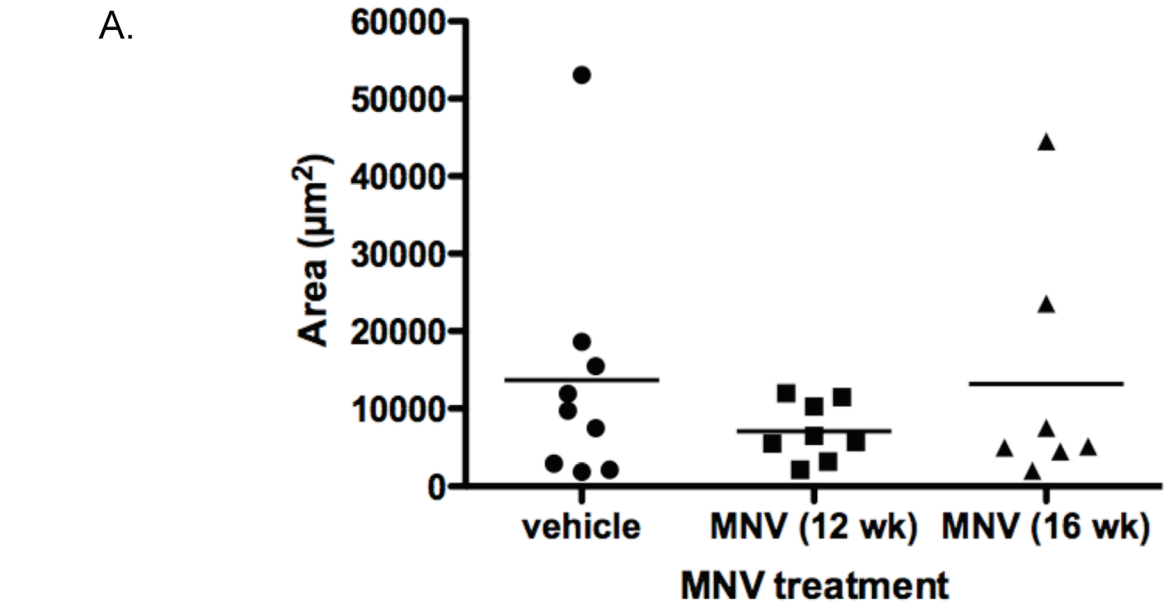


Figure 4. Lipid contents of liver were determined at the end of the study period (16 wk) from mice fed a HFHS diet. (A) Total triglycerides and (B) cholesterol levels did not change due to MNV infection status or timing of the infection (one-way ANOVA).

MNV infection was carried out as described in our previous report.[18] Briefly, MNV4 stocks (gift from Dr Lela Riley, University of Missouri) were propagated in RAW 264.7 cells.[12] Mice were inoculated with MNV (5×10^6 pfu in 200 µL PBS) or vehicle by gavage. Infection status was determined by fecal RT-PCR at the start of the study, 2 wk after MNV infection, and at the end of the study.[12]

Fasting glucose, intraperitoneal glucose tolerance test (IPGTT), and intraperitoneal insulin tolerance test (IPITT). For all procedures, mice were fasted overnight. Fasting blood glucose was determined every 4 wk (study 1) or every 8 wk (study 2) and IPGTT and IPITT at 10 and 11 wk after diet initiation. Glucose concentrations were determined from tail blood by using an automated glucometer (OneTouch Ultra, Lifescan, Milpitas, CA). For IPGTT and IPITT, blood glucose was measured just before intraperitoneal injection of glucose (1 g/kg in sterile PBS) or insulin [1 U/kg Humulin R (Lilly, Indianapolis, IN) in sterile saline] and at 15, 30, 60, and 90 min (for IPITT) or 120 min (for IPGTT) after injection.

Tissue collection. At the end of the study, mice were fasted overnight, weighed, and tested for blood glucose. Mice then were euthanized by CO_2 asphyxiation, followed by cardiocentesis to obtain serum. Livers were weighed and snap-frozen in liquid nitrogen for lipid measurement. Four major adipose depots (epididymal, mesenteric, retroperitoneal, and inguinal fat pads) were dissected and individually weighed to determine adiposity (combined fat-pad weight / body weight × 100%). Hearts and aortas were dissected and fixed in formalin for morphologic analyses.



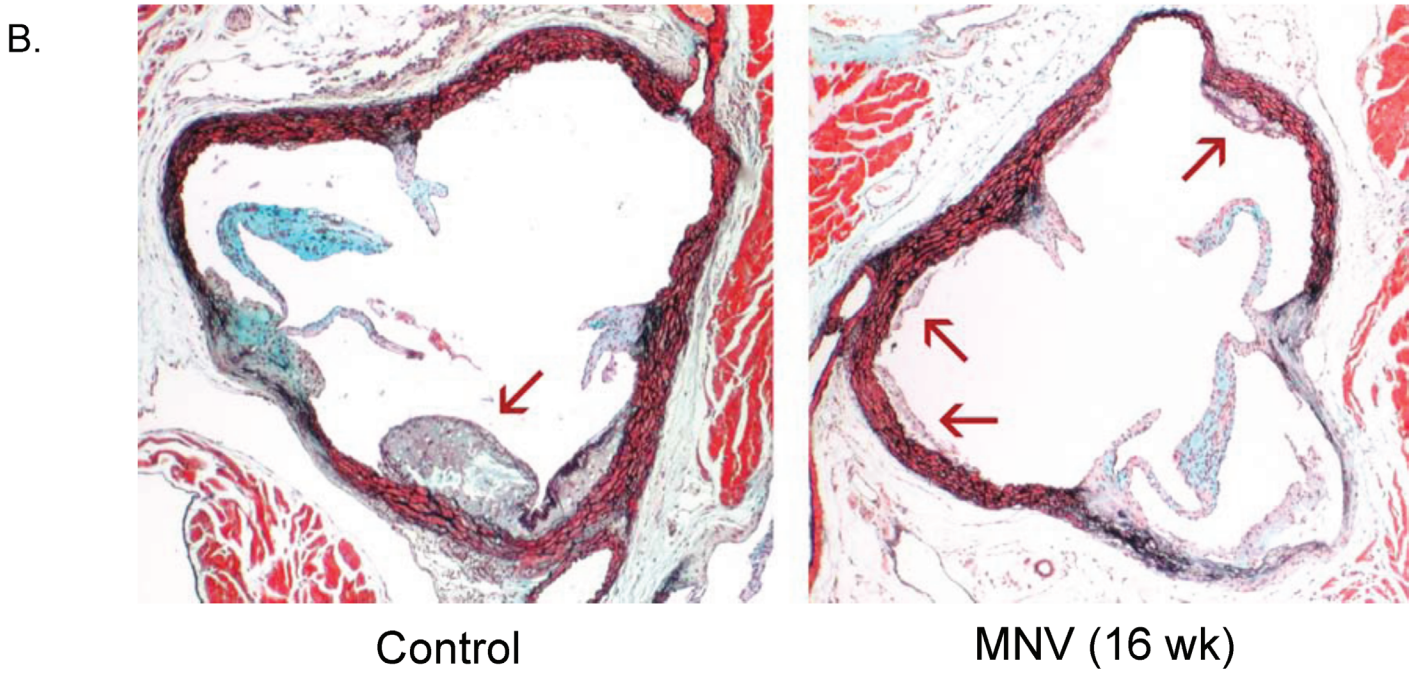


Figure 5. Aortic sinus lesion was evaluated by using Movat pentachrome staining on serial sections of hearts of *Ldlr*$^{-/-}$ mice fed a HFHS diet. (A) Lesion area was determined by using computer-assisted morphometry and was not different between MNV-infected and -uninfected mice (one-way ANOVA). (B) Examples of the most severe lesion (arrows) found in uninfected control and MNV-infected (16 wk) mice. Magnification, 100×.

Serum lipids and insulin. Serum insulin was measured by using ELISA kits (Millipore, MA), and cholesterol and triglycerides were determined by using colorimetric kits (Diagnostic Chemicals Limited, Oxford, CT, and Trig-GB kit, Roche Diagnostics, Mannheim, Germany). Liver lipid levels were determined by using Folch extraction,[7] followed by colorimetric assays as previously.

Quantitation of atherosclerosis. Atherosclerotic lesion areas in the aortic root were quantified as described,[17] with modifications. After overnight fixation in 10% neutral-buffered formalin, the upper sections of the hearts were embedded in paraffin. For each animal, aortic root lesion areas were quantified by an investigator blinded to treatment group from digital photomicrographs by using Image Pro Plus (Version 6.0, Media Cybernetics, Bethesda, MD) from 5 sections stained with Movat pentachrome stain and which spanned 400 μm of the aortic root. For analysis of aortic lesion, aorta was longitudinally split and pinned onto black wax. A photograph of the aorta was taken, and lesion area was measured

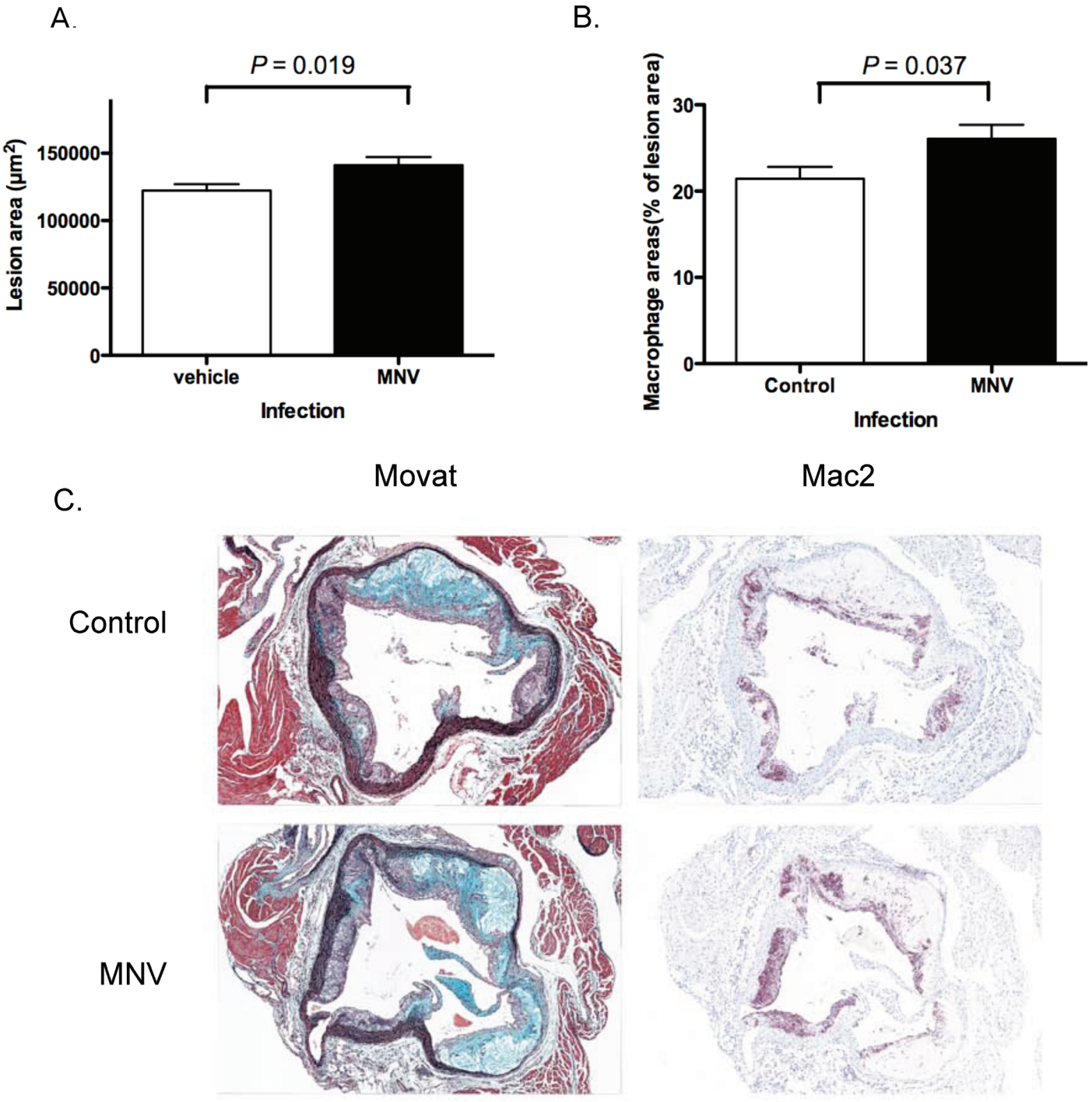


Figure 6. Aortic sinus lesion area and macrophage content were analyzed by using Movat pentachrome staining and immunohistochemistry on serial sections of hearts of *Ldlr*$^{-/-}$ mice fed a HFHC diet. (A) Aortic sinus lesion area was significantly (*t* test, $P = 0.019$) larger in *Ldlr*$^{-/-}$ mice infected with MNV at 8 wk after diet initiation than in sham-infected (vehicle) mice. (B) Immunohistochemistry using Mac2 antibody was performed to determine macrophage content in the lesion. Macrophage area was quantified by computer-assisted morphometry and expressed as percentage of lesion area. MNV-infected mice showed significantly (*t* test, $P = 0.037$) greater macrophage area in the aortic sinus lesion compared with that in vehicle-infected control mice. (C) Representative sections of Movat- and Mac2-stained aortic sinus lesions. Lesion area was determined from Movat-stained tissue sections (4 sections per animal). Mac2 staining (red) was performed to determine macrophage content in the lesion area. Magnification, 100×.

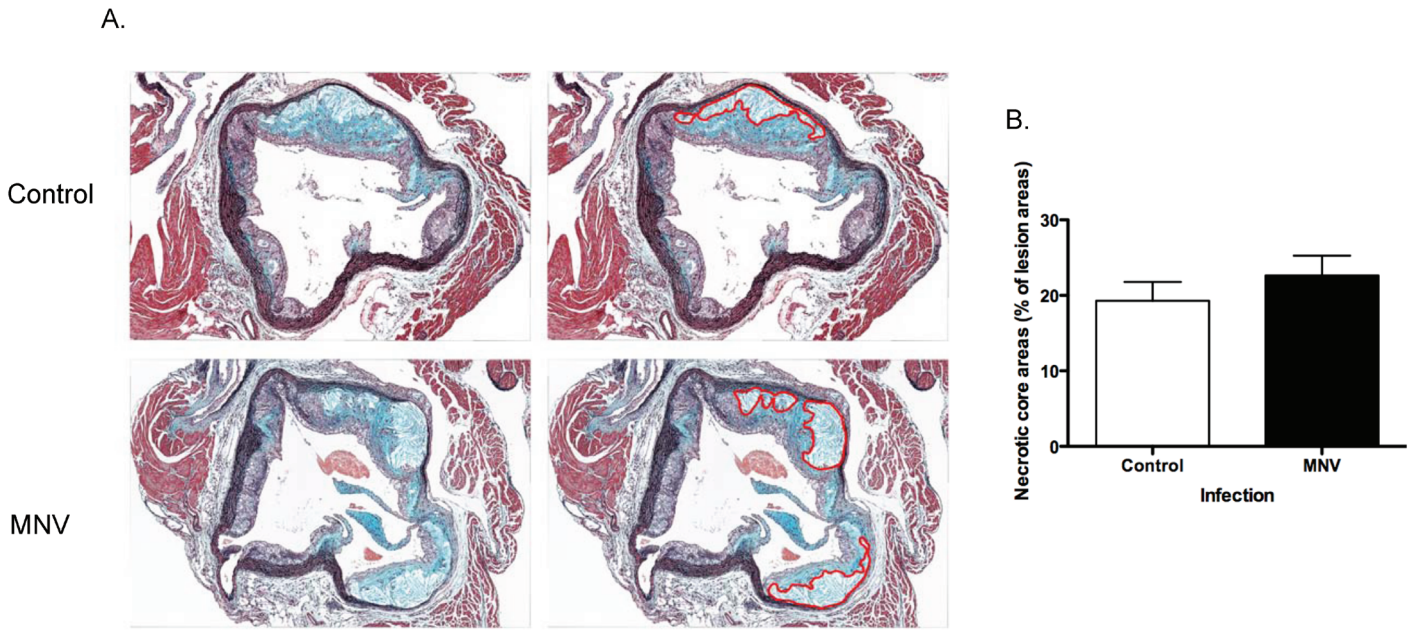


Figure 7. Necrotic core area was determined from Movat-stained tissue sections. (A) From Movat-stained sections (left panels), necrotic core (largely lipid; outlined in red) was identified by using morphometry (right panels) and the percentage of lesion area was calculated. (B) MNV infection did not influence degree of necrosis (*t* test).

using Image J (NIH, Bethesda, MD) and expressed as a percentage of the total area of the aorta.

Compostition of atherosclerotic lesion. Lesion characteristics were quantified by an investigator blinded to treatment groups and using computer-assisted morphometry (ImagePro Plus). Findings were expressed as a percentage of lesion area. Necrotic core areas, defined as areas primarily composed of lipids with cholesterol clefts, cellular debris, and foam cells, were determined from Movat-stained sections.

Macrophage immunohistochemistry. The rat monoclonal antibody Mac2 (titer = 1:2,500; catalog no. CL8942B-3, Cedarlane Laboratories, Burlington, ON) was used to detect aortic sinus lesion macrophages, by using methods described previously.[22,23] Briefly, tissue sections were deparaffinized with xylene, hydrated with graded alcohols, blocked with 3% H_2O_2, washed with PBS, incubated in antigen retrieval solution (catalog no. S1699, Target Retrieval Solution, Dako, Glostrup, Denmark) for 10 min, washed with PBS, incubated with the primary antibody for 60 min, and then washed again with PBS. A biotin-labeled antirat secondary antibody was applied for 30 min, followed by an avidin–biotin–peroxidase conjugate (ABC Elite, Vector Laboratories, Burlingame, CA) for 30 min. A standard peroxidase enzyme substrate, NovaRED (catalog no. SK4800, Vector Laboratories), was added to yield a red reaction product.

Statistics. Data was analyzed by using Prism statistical software (GraphPad Software, La Jolla, CA). One-way ANOVA with a posthoc test (Bonferroni correction) was used for comparison between 3 groups, whereas 2-tailed *t* test was performed for comparison between 2 groups. When repeated measurements were performed on the same animals (IPGTT, IPITT), 2-way ANOVA was used to determine whether significant differences occurred between treatment groups. Statistical significance was defined as *P* value of less than 0.05.

Results

MNV infection status. MNV infection status of mice was determined throughout the study by fecal PCR at baseline, 2 wk after infection, and at the end of the study. By adhering strictly to standard operating protocols, we successfully limited MNV infection to only the intended treatment groups, as determined by fecal RT-PCR analysis. All MNV-infected mice shed virus until the end of the study period, with the longest infection duration being 16 wk (study 1).

Effect of MNV infection on weight gain and glucose metabolism. *Ldlr*$^{-/-}$ mice gained weight steadily throughout the study, achieving approximately 180% (HFHC diet) and 230% (HFHS diet) of initial weight at the end of the diet period (16 wk), regardless of MNV infection status. In addition, adiposity determined from weights of 4 major adipose depots at necropsy did not differ among the treatment groups in both studies (data not shown). Fasting blood glucose levels increased with weight gain and were not influenced by MNV infection status for either diet at all time points (Figure 1). Glucose tolerance and insulin sensitivity did not differ between MNV-infected and uninfected groups in either study (Figure 2). We determined food intake for a period of 1 wk immediately after MNV infection to confirm that the viral infection did not influence appetite. MNV infection did not alter food intake in either study (study 1, 3.0 to 3.5 g per mouse daily; study 2, 2.5 to 3.0 g per mouse daily regardless of MNV infection status).

Effect of MNV infection on circulating and liver lipids. Circulating lipids were determined every 4 wk during study 1. Both total triglycerides and cholesterol levels were increased over the 16-wk diet period, independent of MNV infection status (Figure 3). Consistent with this finding, liver lipids determined at the end of the study were not different between MNV infected compared with uninfected mice (Figure 4). For study 2, both circulating and liver lipids were determined at the end of the study and were not

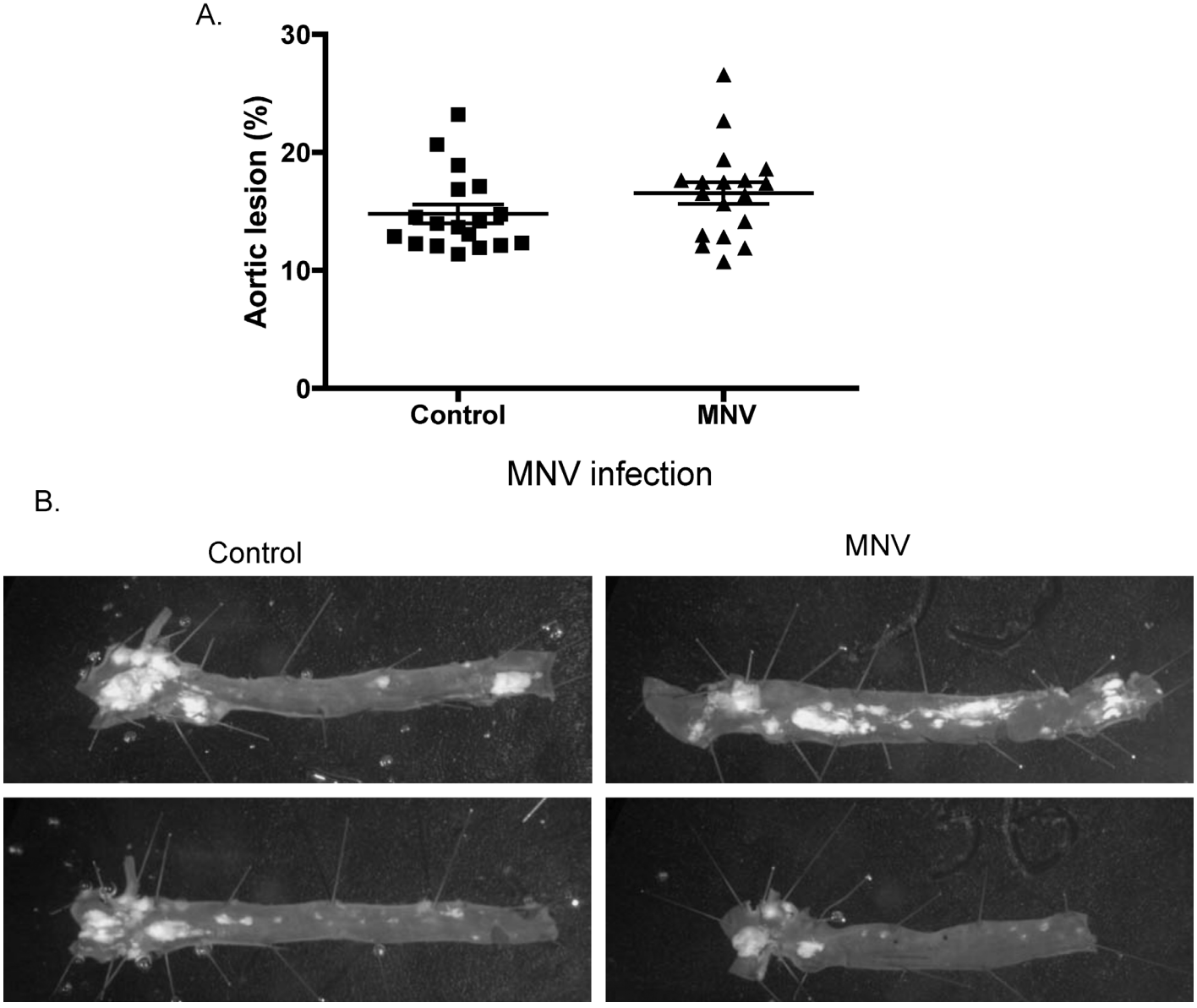


Figure 8. Aorta was opened longitudinally, and lesion area was determined as percentage of aorta. (A) Aortic lesion size was not altered by MNV infection status (*t* test). (B) Two representative aortas from each group are shown. Magnification, 6×.

different between MNV-infected and -uninfected groups (data not shown).

Effect of MNV infection on atherosclerotic lesion area and macrophage accumulation in HFHC diet-fed mice. When fed a HFHS diet, most mice developed small aortic sinus lesions, but atherosclerosis lesion areas were not altered by either MNV infection or by the timing of MNV infection (Figure 5). However, when fed an atherogenic, HFHC diet, all mice developed more severe atherosclerosis, with MNV-infected mice exhibiting a modest but significant ($P < 0.05$) increase in lesion size (16%) as compared with that of uninfected controls (Figure 6 A and C). During the review of Movat pentachrome staining, we noticed some qualitative differences in cellular composition and therefore used Mac2 immunohistochemistry to determine the area of macrophage infiltration in the lesion. Even after adjustment for lesion size, there was a 20% increase in macrophage content of lesions from MNV-infected mice compared with uninfected controls (Figure 6 B and C). To determine whether increased macrophage content was associated with the necrotic core, we measured the core area in the lesions identified with Movat staining (left panels, Figure 7) by using computer-assisted morphometry[4] (right panels, Figure 7). When adjusted for lesion size, total necrotic core area did not differ between the MNV-infected and uninfected groups.

We further evaluated potential effects of MNV infection on atherosclerosis severity in whole aortas by using en face analysis (Figure 8). Most of the plaque was found in the proximal aorta, although in a few cases, a more diffuse plaque buildup could also be seen (Figure 8 B). Total aortic lesion areas, as determined by the en face method, did not differ between MNV-infected and uninfected groups (Figure 8 A).

Discussion

Inflammation plays an important role in the development of obesity, diabetes and atherosclerosis, and the role of macrophages in these diseases is well documented.[19,20,28,30,31] MNV is a subclinical and prevalent virus in many research facilities throughout the United States and is reported to persistently infect macrophages and dendritic cells. Because macrophages and dendritic cells are pivotal in many disease processes and because, at present, MNV is not routinely excluded from SPF mouse facilities, MNV could create a potential confounding variable in disease models. MNV was thought initially to affect only mice with compromised innate immunity. However we have shown that this virus may alter some immune responses even in immunocompetent mice.[18,24] Given its effects on immune responses and its tropism toward macrophages, we hypothesized that MNV infection may alter development and progression of inflammatory diseases in which these immune cells play crucial roles, such as obesity, diabetes, and atherosclerosis.

Our previous report showed that MNV infection does not alter overt disease phenotypes associated with obesity and diabetes in a mouse model of diet-induced obesity (C57BL/6 on a high-fat diet[24]). However, we did note subtle changes in lymphoid tissues in MNV-infected mice in that study. In agreement with our previous report,[24] MNV infection did not result in significant changes in weight gain, fasting blood glucose, glucose tolerance, and insulin sensitivity in *Ldlr*$^{-/-}$ mice fed either diabetogenic or atherogenic diets. These data suggest to us that MNV infection may not significantly alter adipose tissue inflammation and thus insulin resistance.

MNV infection resulted in modest but significant increases in atherosclerotic lesion size and macrophage accumulation in lesions in *Ldlr*$^{-/-}$ mice fed a HFHC diet (study 2) but not in those fed a HFHS diet (study 1). Although all mice fed the HFHS diet developed some degree of aortic sinus atherosclerosis, lesions were significantly smaller compared with what we observed with HFHC feeding. This result was expected, because *Ldlr*$^{-/-}$ mice do not develop severe atherosclerosis without high cholesterol in the diet.[13] However, lesion size varied greatly in both MNV-infected and uninfected mice. Because several mice in study 1 had to be euthanized prior to study end due to ocular trauma after repeated retroorbital bleeding, the study ended with smaller sample sizes than anticipated for each treatment group, with a consequent loss in statistical power. We performed sample size calculation based on our lesion area data to determine how many animals we would need to detect 15% increase in lesion size. Even excluding outliers to reduce variance, we would need a minimum of 128 animals per group to detect 15% increase in lesion size with 95% confidence and 80% power.

For the second study, we reduced the frequency of blood sampling, increased sample size per treatment group, and focused on atherosclerosis as the primary endpoint. Consumption of the atherogenic diet induced larger lesions than those induced by the diabetogenic diet in *Ldlr*$^{-/-}$ mice. In addition, MNV infection was associated with significantly increased lesion size and increased macrophage accumulation. Because MNV infection occurred 8 wk after diet initiation, when the atherosclerotic lesion would be actively developing, we hypothesized that MNV may accelerate the plaque buildup by promoting macrophage infiltration in the lesion. Supporting our hypothesis, a recent report shows that endocytosis of MNV into macrophages is dependent on cholesterol and dynamin II, 2 factors that are important in atherosclerosis development.[26]

We performed 2 assays to determine effects of MNV on atherosclerosis: Movat staining and lesion area calculation in aortic sinus and atherosclerotic lesion area in en face aorta. MNV was associated with increased lesion size in the aortic sinus but not the aorta. On the basis of our current study, we are uncertain why this difference occurs. Perhaps MNV influences the development of atherosclerosis in different anatomic areas (aortic sinus compared with aorta). Alternatively, the en face method may not be sufficiently sensitive to detect subtle changes in lesion size, because 3D information is lost when you measure the surface area of the plaque.

Our results are not unique, given that other infectious agents including virus[6,8] and bacteria[1,15] have been shown to participate in the development of atherosclerosis. However, the current study is the first demonstration that a 'subclinical' and prevalent virus in SPF rodent research facilities can alter atherosclerosis progression in a frequently used murine model, *Ldlr*$^{-/-}$ mice. In our previous study using C57BL/6 mice, we reported that MNV-infected mice seroconverted and shed virus until the study end point at 10 wk after infection.[24] In the current studies, *Ldlr*$^{-/-}$ mice, which are on a C57BL/6 background, shed MNV in feces for as long as 16 wk after MNV infection, indicating that MNV is a persistent infectious agent that may be difficult to eradicate from mouse colonies. Nonetheless, by demonstrating a measurable effect of MNV infection on lesion size in a commonly studied murine atherosclerosis model, our study demonstrates that MNV is an intercurrent variable in studies of atherosclerosis in *Ldlr*$^{-/-}$ mice and suggests that the virus may need to be eliminated from mouse colonies of this strain intended for atherosclerosis research.

Acknowledgments

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