

## Original Research

# Murine Norovirus Infection Variably Alters Atherosclerosis in Mice Lacking Apolipoprotein E

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Macrophages play a key role in the development of atherosclerosis. Murine noroviruses (MNV) are highly prevalent in research mouse colonies and infect macrophages and dendritic cells. Our laboratory found that MNV4 infection in mice lacking the LDL receptor alters the development of atherosclerosis, potentially confounding research outcomes. Therefore, we investigated whether MNV4 likewise altered atherosclerosis in ApoE<sup>-/-</sup> mice. In the presence of oxidized LDL, MNV4 infection of ApoE<sup>-/-</sup> bone marrow-derived macrophages increased the gene expression of the inflammatory markers inducible nitric oxide synthase, monocyte chemoattractant protein 1, and IL6. In addition, proteins involved in cholesterol transport were altered in MNV4-infected ApoE<sup>-/-</sup> bone marrow-derived macrophages and consisted of increased CD36 and decreased ATP-binding cassette transporter A1. MNV4 infection of ApoE<sup>-/-</sup> mice at 12 wk of age (during the development of atherosclerosis) had a variable effect on atherosclerotic lesion size. In one study, MNV4 significantly increased atherosclerotic plaque area whereas in a second study, no effect was observed. Compared with controls, MNV4-infected mice had higher circulating Ly6C-positive monocytes, and viral RNA was detected in the aortas of some mice, suggesting potential mechanisms by which MNV4 alters disease progression. Plaque size did not differ when ApoE<sup>-/-</sup> mice were infected at 4 wk of age (early during disease development) or in ApoE<sup>-/-</sup> mice maintained on a high-fat, high-cholesterol diet. Therefore, these data show that MNV4 has the potential to exert a variable and unpredictable effect on atherosclerosis in ApoE<sup>-/-</sup> mice. We therefore propose that performing experiments in MNV-free mouse colonies is warranted.

**Abbreviations:** ABCA1, ATP-binding cassette transporter A1; ApoE, apolipoprotein E; BMDM, bone-marrow-derived macrophages; iNOS, inducible nitric oxide synthase; LDLr, low-density lipoprotein receptor; MCP1, monocyte chemoattractant protein 1; MNV, murine norovirus; oxLDL, oxidized LDL.

Atherosclerosis is an inflammatory disease resulting in the hardening and narrowing of arteries leading to an increased incidence of heart attack and stroke in humans. Mouse models have been useful in understanding and characterizing atherosclerotic plaque development.<sup>23</sup> Wild-type mice are relatively resistant to atherosclerosis and generally require genetic manipulation to increase their susceptibility.<sup>23</sup> The 2 most commonly used models of atherosclerosis are mice deficient in either apolipoprotein E (ApoE) or the LDL receptor (LDLr). Both the ApoE<sup>-/-</sup> and LDLr<sup>-/-</sup> strains are dyslipidemic due to impaired lipoprotein production and metabolism, resulting in susceptibility to atherosclerosis.<sup>15,23</sup> Whereas LDLr<sup>-/-</sup> mice develop pronounced atherosclerosis only after consuming an atherogenic (high-fat, high-cholesterol) diet, ApoE<sup>-/-</sup> mice spontaneously develop disease without dietary intervention.

There is evidence that infectious agents play a role in atherosclerosis in both humans and in animal models.<sup>17,69,76</sup> Both bacterial and viral agents have been reported to alter pathogenic mechanisms of atherosclerosis and to increase the risk of cardiovascular disease. These agents include *Chlamydia pneumoniae*,<sup>5,8,10,51</sup>

*Porphyromonas gingivalis*,<sup>27,69</sup> HIV,<sup>7,14,54,71</sup> cytomegalovirus,<sup>8,31,65</sup> and influenza virus.<sup>25,47,57</sup> Our laboratory recently reported that murine norovirus (MNV) infection increases the atherosclerotic lesion area and macrophage content in the aortic sinus of LDLr<sup>-/-</sup> mice fed a high-fat, high-cholesterol diet compared with uninfected mice.<sup>62</sup> First reported in 2003, MNV is a highly infectious, environmentally stable, nonenveloped, RNA virus in the family *Caliciviridae* and the genus *Norovirus*.<sup>38</sup> MNV infection in most strains of laboratory mice results in persistent infections and chronic fecal shedding without overt clinical signs of disease.<sup>34,75</sup> These attributes of the virus contribute to its high prevalence of infection—as high as 32%—reported in large-scale surveys of laboratory mice worldwide.<sup>35,66</sup> Notably, MNV has a tropism to infect macrophages and dendritic cells<sup>81</sup> and therefore has the potential to alter scientific studies in laboratory mice in which macrophages and other inflammatory cells play an important role in the immune responses, disease progression, and pathogenesis of disease. For example, monocytes and macrophages are critical components in the development of atherosclerosis.<sup>36,52,73</sup> Therefore, in the current study, our laboratory sought to expand our findings regarding the effect of MNV infection on aortic lesion size in LDLr<sup>-/-62</sup> mice by determining whether MNV infection likewise affects atherosclerosis development in another frequently used mouse model, ApoE<sup>-/-</sup> mice.

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We hypothesized that MNV increases atherosclerotic lesion formation in ApoE<sup>-/-</sup> mice, potentially by altering inflammatory cytokines and chemokines, by shifting macrophage subsets or numbers, or by modifying the formation of foam cells via changes to cholesterol efflux or uptake mechanisms. We demonstrate here that MNV infection alters inflammatory cytokine production by macrophages and modulates proteins associated with cholesterol transport in macrophages in vitro. In vivo studies in ApoE<sup>-/-</sup> mice revealed that MNV infection increased atherosclerotic lesion size, but this effect was variable. We therefore conclude that MNV infection, specifically the MNV4 isolate used in the current study and previously in LDLR<sup>-/-</sup> mice,<sup>62</sup> has the potential to exert a variable and unpredictable effect in mouse models of atherosclerosis.

## Materials and Methods

**Animals and husbandry.** Male ApoE<sup>-/-</sup> mice (B6.129P2-ApoE<sup>tm1Unc</sup>/J) were obtained from The Jackson Laboratory (Bar Harbor, ME) and acclimated for 1 wk prior to study initiation. Mice were certified by the vendor to be free of specific rodent pathogens including ectoparasites, endoparasites, *Pneumocystis murina*, *Helicobacter* spp., known enteric and respiratory bacterial pathogens, and antibodies to murine norovirus, mouse hepatitis virus, Sendai virus, pneumonia virus of mice, reovirus 3, Theiler murine encephalomyelitis virus, ectromelia virus, polyoma virus, lymphocytic choriomeningitis virus, mouse adenovirus, minute virus of mice, mouse parvovirus, mouse rotavirus, mouse cytomegalovirus, mouse thymic virus, Hantaan virus, K virus, *Encephalitozoon cuniculi*, cilia-associated respiratory bacillus, *Mycoplasma pulmonis*, and *Clostridium piliforme*. With the exception of experimental infection with murine norovirus, mice were maintained free of the listed pathogens throughout the study on the basis of sentinel surveillance of the animal housing room and through the use of the procedures described. ApoE<sup>-/-</sup> mice were group-housed by experimental infection status in autoclaved, individually ventilated cages (Thoren, Hazleton, PA) with corncob bedding (The Andersons, Maumee, OH) and provided acidified, reverse-osmosis-purified, autoclaved water in bottles. All manipulations were performed in a vertical-flow animal-transfer station (AniGard II, The Baker Company, Sanford, ME) disinfected with chlorine dioxide (Clidox S, 1:18:1 dilution, Pharmacal Research Laboratories, Naugatuck, CT). The University of Washington's animal facilities are AAALAC-accredited, and all animal studies were approved by the University of Washington's IACUC.

**Experimental infections with MNV4.** MNV4 was propagated in RAW 264.7 cells and plaque-assayed as previously described,<sup>35</sup> with the modifications of using 1% penicillin-streptomycin instead of ciprofloxacin and no HEPES. Two viral propagations were used for the studies. Clarified supernatants (2000 × g, 15 min, 4 °C) containing MNV4 virus of unknown passage number were used for in vitro inflammatory gene expression, CD36, and ATP-binding cassette transporter A1 (ABCA1) experiments, whereas ultracentrifugation (100,000 × g, 3 h, 4 °C) of the same MNV4 virus of unknown passage and resuspended in PBS was used for the first in vivo experimental infection in ApoE<sup>-/-</sup> mice maintained on a normal chow diet. All subsequent in vivo experiments were performed by using the clarified supernatant of another preparation of low-passage (passage 7) MNV4. Clarified supernatants of uninfected RAW 264.7 cells, or ultracentrifuged uninfected RAW 264.7 cells resuspended in PBS, were used for control inoculations.

To evaluate atherosclerosis development in ApoE<sup>-/-</sup> mice maintained on a normal chow diet, 4-wk-old, male ApoE<sup>-/-</sup> mice were fed a standard irradiated rodent chow (Purina Lab Diet 5053, Brentwood, MO) free choice for the duration of the study. In the first study, mice were orally gavaged at 12 wk of age with 1 × 10<sup>6</sup> pfu of MNV4 (*n* = 19), whereas control mice were orally gavaged with uninfected RAW 264.7 lysate (*n* = 17); all mice then were maintained an additional 9 wk until study termination when they were 21 wk old. In the second study, to evaluate the timing of MNV4 infection in the development of atherosclerosis, one group of mice was orally gavaged with 1 × 10<sup>6</sup> pfu of MNV4 (*n* = 19) at study start (when mice were 4 wk old), whereas another subset of mice were infected at 12 wk of age (*n* = 20), similar to the conditions of the first study. Control mice were orally gavaged with uninfected RAW 264.7 lysate (*n* = 18) at both corresponding time points, and all mice were maintained until study termination, when they were 20 wk old.

To evaluate atherosclerosis development in ApoE<sup>-/-</sup> mice maintained on a western-style (high-fat, high-cholesterol) diet and to evaluate MNV4 spread to tissues by using RT-PCR, 4-wk-old, male ApoE<sup>-/-</sup> mice were fed a high-fat, high-cholesterol, irradiated diet (no. TD88137, Harlan, Indianapolis, IN) for 3 wk to ensure the formation of atherosclerotic lesions at the time of infection. At 7 wk of age, mice were orally gavaged with 1 × 10<sup>6</sup> pfu of MNV4. Tissue samples were evaluated by RT-PCR at 1 and 2 wk after infection (*n* = 12 mice per time point) to assess viral spread and by histopathology at 8 wk after infection for the development of atherosclerosis (*n* = 22 mice per group).

For all studies, at study termination, mice were euthanized by using an inhaled overdose of CO<sub>2</sub>. Infection status was confirmed by fecal RT-PCR. Blood was collected by cardiocentesis, and tissues for RT-PCR were collected aseptically. For aortic sinus plaque analysis, hearts and aortas were dissected and fixed in formalin after PBS perfusion.

**Flow cytometry.** Heparinized blood (20 U heparin per 1 mL blood) was collected by cardiocentesis 1 wk after infection from mice dosed with MNV4 at 12 wk of age. RBC were lysed in Gey's solution. Splenic single cell suspensions were made as previously described<sup>6</sup> from mice inoculated with MNV4 at 12 wk old and collected at 1 wk or 9 wk after infection. Cellularity was determined by using a hemocytometer. Nonspecific binding by cells was blocked by using antiCD16/CD32 (BD Biosciences, San Jose, CA), which then were stained with antibodies to distinguish neutrophils, monocytes, macrophages, and dendritic cells similar to previously described.<sup>67</sup> Antimouse antibodies were purchased from BD Biosciences, eBioscience (San Diego, CA), or Biolegend (San Diego, CA) for the following cell surface markers: the lineage (Lin) markers NK1.1, CD90.2, B220, Ly6G, Ter119, and CD49b or NK1.1, CD90.2, B220, and Ly6G as well as Ly6C, CD11b, CD11c, I-A/I-E, and F4/80. Single cells were gated by using the forward-scatter A and W parameters, and leukocytes were gated on forward-scatter A and side-scatter A for size. Lin-stained cells were further separated to identify neutrophils (Lin<sup>+</sup>CD11b<sup>+</sup>F4/80<sup>-</sup> class II<sup>-</sup>CD11c<sup>-</sup>), macrophages or dendritic cells (Lin<sup>-</sup>CD11b<sup>+</sup> class II<sup>+</sup>), macrophages (Lin<sup>-</sup>CD11b<sup>+</sup> class II<sup>+</sup>F4/80<sup>+</sup>), or monocytes (Lin<sup>-</sup>CD11b<sup>+</sup>F4/80<sup>-</sup> class II<sup>-</sup>CD11c<sup>-</sup>). Monocytes were further characterized according to Ly6C expression. ApoE<sup>-/-</sup> bone-marrow-derived macrophages (BMDM) were blocked with antiCD16/CD32 (BD Biosciences) and evaluated for the expression of CD36 by using a monoclonal antibody against CD36 (clone no.

72-1, APC conjugate, eBioscience, San Diego, CA). Data were collected on a LSRII or FACS Canto II (BD Biosciences) and analyzed by using FlowJo (Tree Star, Ashland, OR).

**Western blot analysis.** Protein concentrations were determined by using BCA protein assay reagent (Thermo Scientific, Waltham, MA). Total protein (15 to 20 µg per lane) was separated on a gradient (4% to 15%) denaturing gel (BioRad, Hercules, CA) and transferred onto polyvinylidene difluoride membrane (BioRad) with 1× CAPS buffer. The membrane was incubated with ABCA1 antibody (polyclonal; Novus Biologic, Littleton, CO) in 5% BSA overnight, washed with TBST, and incubated with anti-rabbit secondary antibody conjugated with horseradish peroxidase (Thermo Scientific). Signal was detected by using SuperSignal West Femto Chemiluminescent Substrate (Thermo Scientific). For a loading control, GAPDH was detected on the same blot (GAPDH antibody G9545, Sigma-Aldrich, St Louis, MO).

**Quantitation of atherosclerosis and macrophage immunohistochemistry.** Aortic sinus sections were stained with Movat pentachrome stain, and lesion area was determined as previously described.<sup>62</sup> Macrophage infiltration in aortic lesions was determined by immunohistochemistry using a monoclonal rat antimouse Mac2 antibody (Cedarlane Laboratories, Burlington, NC) as previously described<sup>62</sup> with slight modifications. Modifications included blocking with normal horse serum and using an ImmPRESS HRP Ig secondary antibody (Vector Laboratories, Burlingame, CA) and ImmPACT NovaRED Peroxidase Substrate (Vector Laboratories).

**Serum cholesterol and cytokine expression.** The following assays were performed according to the manufacturer's recommended protocols. Serum cholesterol was measured by using colorimetric kits (Diagnostic Chemical Limited, Oxford, CT). Serum cytokine and chemokine levels were analyzed in duplicate by using the BioPlex Pro Mouse Cytokine 23plex Assay kit (BioRad) or the Milliplex Mouse Cytokine/Chemokine 22plex kit (Millipore, Billerica, MA) and run on a BioPlex 200 System with BioPlex Manager software version 5.0 (BioRad). Cytokines evaluated included IL1α, IL1β, IL2, IL3, IL4, IL5, IL6, IL7, IL9, IL10, IL12p40, IL12p70, IL13, IL15, IL17, IL17A, eotaxin, G-CSF, GM-CSF, IP10, IFNγ, KC, monocyte chemoattractant protein 1 (MCP1), MIP1α, MIP1β, RANTES, and TNFα. Serum levels of sE-selectin, MMP9, sICAM1, Pecam1, and total PAI1 were evaluated in duplicate by using the Milliplex Cardiovascular Disease Panel 1 kit (Millipore).

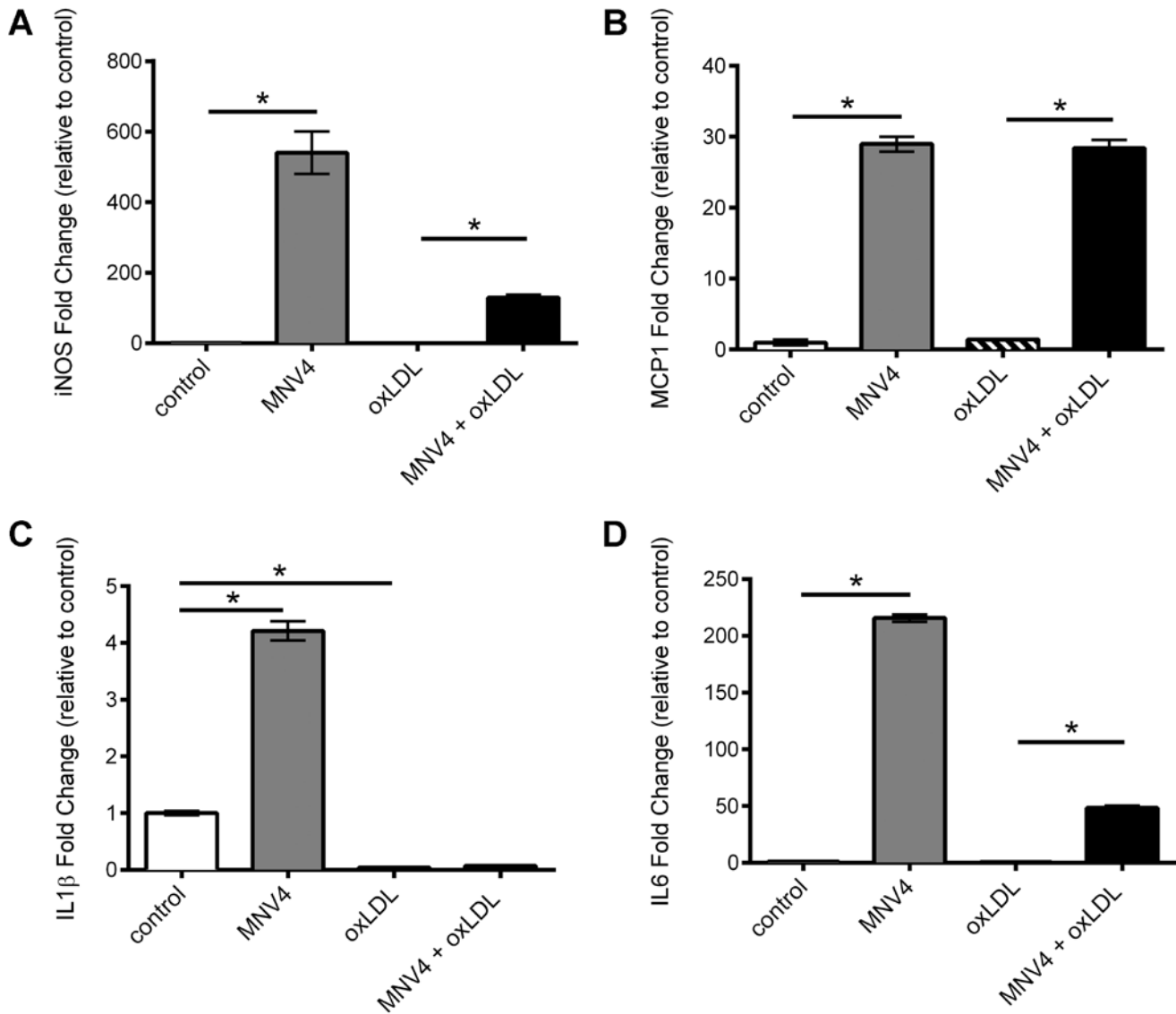
**RT-PCR assay for MNV4.** Primers to detect MNV have been described previously.<sup>33,34</sup> The following procedures were performed according to manufacturer's recommended protocols. Tissue and fecal samples were homogenized in RLT buffer for 30 or 40 s by using Lysing Matrix D or E tubes and the FastPrep24 kit (MP Biomedicals, Solon, OH), centrifuged at 17,000 × g for 3 min, and RNA extracted from the resulting supernatant by using the RNeasy Mini Kit (Qiagen, Valencia, CA). Bone marrow samples were homogenized in RLT buffer by using QIAshredder columns (Qiagen). RNA was extracted from unstained, paraffin-embedded, cross-sections of aortic sinus (thickness, 4-µm) scraped off glass slides (3 sections pooled per animal) by using the RNeasy FFPE Kit (Qiagen). The QIAmp Viral RNA Mini Kit (Qiagen) was used to extract RNA from the clarified supernatants of MNV4-infected RAW 264.7 cells. RT-PCR for MNV was performed on 2 or 5 µL of RNA template in a 25- or 50-µL reaction volume by using

the OneStep RT-PCR Kit (Qiagen) with thermocycling parameters as previously described.<sup>33,34</sup> RT-PCR products were purified from agarose gels by using the Wizard SV Gel and PCR Clean-Up System (Promega, Madison, WI).

**In situ hybridization for MNV4.** In situ hybridization was performed with digoxigenin-labeled cRNA probes. An RT-PCR product was amplified as described earlier from MNV4 RNA by using the primers MNV forward (5' AAT TAA CCC TCA CTA AAG GG T TTG GAA CAA TGG ATG CTG A3') and MNV reverse (5' TAA TAC GAC TCA CTA TAG GG A GTG GTG AGT GAC CCT TTG G 3'), with sequences for RNA polymerases T3 and T7 (shown underlined) appended. Gene-specific antisense and sense cRNA probes were synthesized by in vitro transcription of DNA with Digoxigenin RNA Labeling Mix (Roche Diagnostics, Indianapolis, IN). Formalin-fixed, paraffin-embedded tissue sections were deparaffinized and digested with proteinase K, and a digoxigenin-labeled cRNA probe was hybridized at 55 °C for 16 h. Visualization of mRNA was performed with alkaline-phosphatase-conjugated antidigoxigenin antibody (Roche Diagnostics) by using 4-nitroblue tetrazolium chloride and 5-bromo-4-chloro-3-indolyl phosphate (Roche Diagnostics). Controls included digoxigenin-labeled sense cRNA probe and uninfected tissues; no specific signal was seen in these negative controls.

**BMDM.** Bone marrow cells were isolated from ApoE<sup>-/-</sup> mice (age, 10 to 12 wk) and macrophages differentiated in cell culture for 7 to 10 d, as previously described.<sup>42</sup> Differentiated macrophages were plated at 1 to 1.4 × 10<sup>6</sup> cells per well in 6-well plates. ApoE<sup>-/-</sup> BMDM were infected with MNV4 in triplicate wells at a multiplicity of infection of 0.2 with or without oxidized LDL (10 µg/mL, Intracel Resources, Frederick, MD). Oxidized LDL was added to mimic the hypercholesterolemic environment found in the blood of ApoE<sup>-/-</sup> mice. Clarified RAW 264.7 lysate was used for uninfected controls. After incubation for 24 h, RNA extraction and gene expression analysis by real-time RT-PCR was performed as previously described.<sup>42</sup> Target gene expression was normalized to the housekeeping gene hypoxanthine-guanine phosphoribosyltransferase<sup>18</sup> and then calculated as the fold change relative to the average value obtained from the no-treatment control. Primers for PCR were: TNFα forward, 5' CTG AAC TTC GGG GTG ATC GG 3'; TNFα reverse, 5' GGC TTG TCA CTC GAA TTT TGA GA 3'; and those previously described for IL6,<sup>50</sup> IL1β,<sup>18</sup> inducible nitric oxide synthase (iNOS),<sup>46</sup> MCP1,<sup>28</sup> IFNβ,<sup>79</sup> CD36,<sup>63</sup> and ABCA1.<sup>63</sup> For western blot analysis, protein was extracted by using MPER Protein Extraction Kit (Thermo Scientific). For flow-cytometric analysis, ApoE<sup>-/-</sup> BMDM were dissociated by cold shock in PBS (Ca<sup>2+</sup>- and Mg<sup>2+</sup>-free).

**Statistics.** Statistical analyses were performed by using Prism version 5 or 6 (GraphPad Software, San Diego, CA). An unpaired Student's *t* test or Mann-Whitney test was used to compare 2 groups, and one-way ANOVA with a Dunnett or Sidak multiple-comparison test was used to compare more than 2 groups. For gene-expression analysis, one-way ANOVA with the Sidak multiple-comparison test was used to compare the following groups: control uninfected BMDM compared with MNV4-infected, control uninfected BMDM treated with and without oxidized LDL (oxLDL), and control uninfected oxLDL-treated BMDM compared with MNV4-infected oxLDL-treated. Pearson correlation was used to evaluate correlation between atherosclerotic lesion size and serum analytes. Statistical significance was defined as a *P* value of less than 0.05.

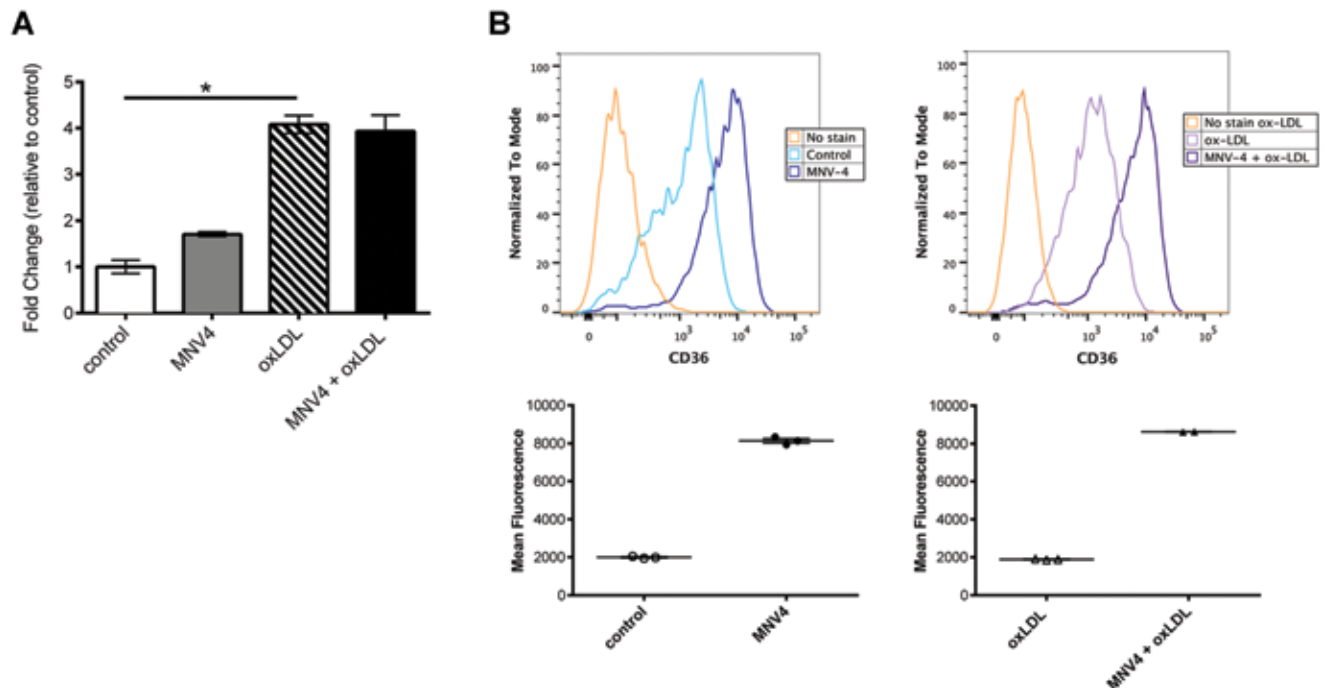


**Figure 1.** MNV4 alters cytokine mRNA expression levels (mean  $\pm$  SEM) in ApoE<sup>-/-</sup> BMDM. Triplicate wells of ApoE<sup>-/-</sup> BMDM were inoculated with MNV4 (multiplicity of infection, 0.2) or uninfected clarified RAW cell lysate (control), with and without oxLDL, and incubated for 24 h. RNA was extracted and evaluated for cytokine gene expression by real-time RT-PCR. Statistical significance (\*,  $P < 0.05$ ) was determined by ANOVA followed by the Sidak multiple-comparisons test. (A) iNOS. (B) MCP1. (C) IL1 $\beta$ . (D) IL6.

## Results

**Effect of MNV4 infection on proinflammatory gene expression in ApoE<sup>-/-</sup> BMDM.** To determine whether MNV infection could induce changes in gene expression of proinflammatory markers, we investigated the effects of MNV4 infection on ApoE<sup>-/-</sup> BMDM in vitro with and without oxidized LDL (oxLDL). MNV4 infection alone significantly ( $P < 0.05$ ) increased expression of iNOS, MCP1, IL1 $\beta$ , and IL6 in ApoE<sup>-/-</sup> BMDM (Figure 1). In the presence of oxLDL, MNV4 infection likewise significantly ( $P < 0.05$ ) increased the expression of iNOS, MCP1, and IL6 (Figure 1). In addition, oxLDL treatment alone decreased the expression of IL1 $\beta$  compared with that in untreated control cells, and MNV4 infection did not alter the expression of IL1 $\beta$  in the presence of oxLDL. Overall, these data indicate that MNV4 alters the gene expression of macrophage proinflammatory markers in the presence or absence of oxLDL in vitro.

**Effect of MNV4 on the expression of proteins involved in cholesterol transport in ApoE<sup>-/-</sup> BMDM.** Altered cholesterol transport in macrophages is associated with increased foam cell formation and may result in increased atherosclerosis.<sup>78,83,85</sup> Furthermore, there is evidence that viral infections or activation of Toll-like receptors can alter cellular cholesterol-efflux transporters, such as ABCA1, or cholesterol-uptake scavenger receptors, such as CD36.<sup>11,19,21,54,70</sup> Because MNV4 altered cytokine and chemokine gene expression in ApoE<sup>-/-</sup> BMDM in the presence of oxLDL and given that RNA viruses can activate Toll-like receptors, we investigated the expression of the CD36 and ABCA1 in MNV4-infected ApoE<sup>-/-</sup> BMDM. Gene expression of CD36 was significantly ( $P < 0.05$ ) upregulated in response to oxLDL treatment alone but was unaltered in response to MNV4 infection regardless of whether oxLDL was present or not (Figure 2 A). Although MNV4 infection did not change CD36 mRNA expression, the



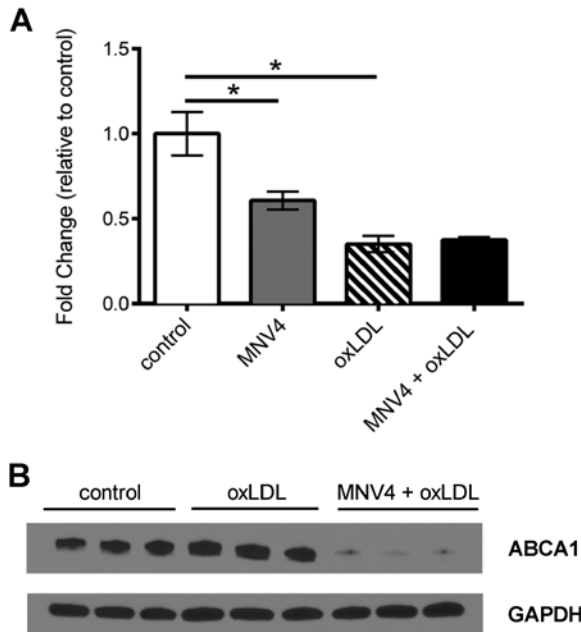
**Figure 2.** MNV4 alters scavenger receptor CD36 protein expression in ApoE<sup>-/-</sup> BMDM. Triplicate wells of ApoE<sup>-/-</sup> BMDM were inoculated with MNV4 (multiplicity of infection, 0.2) or with uninfected clarified RAW cell lysate (control), with and without oxLDL, and incubated for 24 h. (A) RNA was extracted and evaluated for gene expression of CD36 by real-time RT-PCR analysis. Statistical significance (\*,  $P < 0.05$ ) was determined by ANOVA followed by the Sidak multiple-comparisons. (B) Surface protein expression of CD36 evaluated by flow cytometry is increased by MNV4 infection in the absence (left) and in the presence (right) of oxLDL in ApoE<sup>-/-</sup> BMDM. Representative samples are shown in the histograms, and mean fluorescence of triplicate cultures is plotted below histograms. Bars represent mean  $\pm$  SEM.

surface expression of CD36 protein was upregulated when ApoE<sup>-/-</sup> BMDM were infected with MNV4 (Figure 2 B). Gene expression of ABCA1 was significantly ( $P < 0.05$ ) decreased by MNV4 infection alone (that is, control compared with MNV4) as well as by treatment with oxLDL alone (Figure 3 A). In the presence of oxLDL, infection with MNV4 did not alter the gene expression of ABCA1 (Figure 3 A). However, the protein expression of ABCA1 was decreased by MNV4 infection in the presence of oxLDL (Figure 3 B) even though mRNA expression levels were not changed. In summary, these data show that MNV4 infection increased the surface expression of CD36 protein, whereas in the presence of oxLDL, infection decreased the protein expression of ABCA1, and both of these alterations could result in increased intracellular cholesterol content.

**Effect of MNV4 on atherosclerosis in ApoE<sup>-/-</sup> mice fed a normal chow diet.** To determine whether MNV4 infection alters atherosclerotic disease development in ApoE<sup>-/-</sup> mice fed a normal chow diet, mice were infected with MNV4 at 12 wk old and then evaluated at 21 wk old. Infection with MNV4 significantly ( $P = 0.0131$ ) increased aortic sinus lesion area (mean  $\pm$  SEM,  $41,066 \pm 5555 \mu\text{m}^2$ ) by approximately 1.7-fold when compared with uninfected controls ( $23,695 \pm 3249 \mu\text{m}^2$ ; Figures 4 A and 5). In situ hybridization of atherosclerotic plaques for MNV4 revealed positive staining within aortic sinus plaques in 4 of the 15 MNV4-infected mice but not in uninfected controls (Figure 5). In addition, RT-PCR analysis revealed MNV4 RNA in the paraffin sections of aortic sinus from 2 of 19 MNV4-infected mice; all infected mice were confirmed positive for MNV by fecal RT-PCR assay. Despite the increased size of the aortic sinus plaque area in MNV4-infected mice, the percentage macrophage area within the atherosclerotic

lesion evaluated by Mac2 immunohistochemical staining did not differ between MNV4-infected and uninfected mice (Figures 4 B and 5). MNV4 infection did not alter weight gain throughout the study (data not shown). No differences were noted in the serum levels of cholesterol or 23 inflammatory cytokines and chemokines between MNV4-infected and uninfected control mice at study endpoint (data not shown). No differences were found at study endpoint in splenic cellularity or cellular subsets analyzed—including neutrophils, macrophages and dendritic cells, monocytes, and Ly6C-positive monocytes—between MNV4-infected mice and uninfected controls (data not shown). Serum levels of the cardiovascular disease biomarkers sE-selectin, sICAM1, Pecam1, and total PAI1 did not differ (data not shown), whereas MMP9 was significantly ( $P = 0.0262$ ) increased in MNV4-infected mice ( $152.9 \pm 25.3 \text{ ng/mL}$ ) as compared with uninfected controls ( $98.2 \pm 27.4 \text{ ng/mL}$ ). However, the increased serum MMP9 concentration did not correlate with increased atherosclerotic lesion size in individual mice (data not shown).

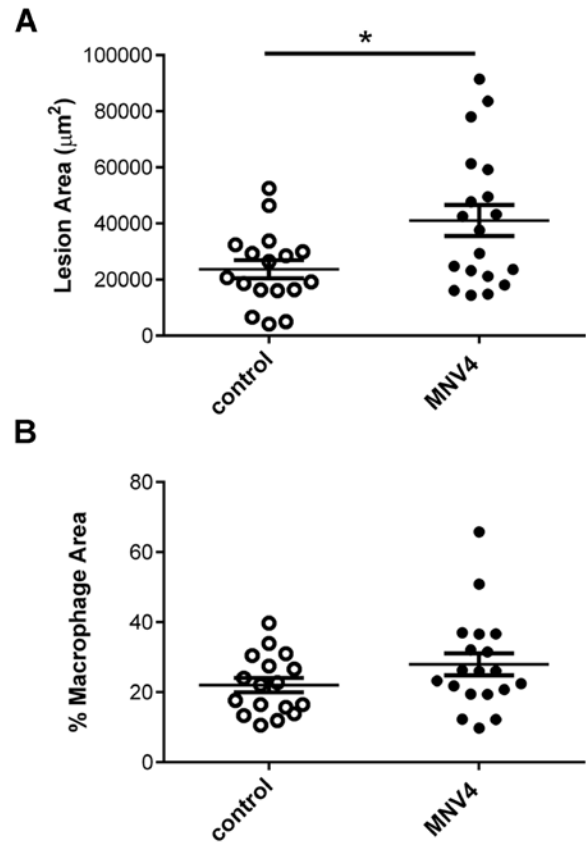
To determine whether the timing of MNV4 infection altered disease development in ApoE<sup>-/-</sup> mice, we performed a second, similar study that contained an additional experimental group of mice infected at 4 wk of age as well as at 12 wk old. These time points were chosen to evaluate the potential effect of MNV infection at the start of a study compared with MNV infection during an ongoing study, in which atherosclerotic plaques are already developed in 12-wk-old mice. All infected mice were confirmed positive for MNV by fecal RT-PCR assay. In addition, the percentage of Ly6C-positive monocytes was significantly ( $P = 0.0287$ ) increased in the blood when a subset of the mice infected with MNV4 at 12 wk of age was evaluated, and this increase was concordant with a



**Figure 3.** MNV4 decreases cholesterol transporter ABCA1 protein expression in ApoE<sup>-/-</sup> BMDM. Triplicate wells of ApoE<sup>-/-</sup> BMDM were inoculated with MNV4 (multiplicity of infection, 0.2) or with uninfected clarified RAW cell lysate (control), with and without oxLDL, and incubated for 24 h. (A) RNA was extracted and evaluated for gene expression of ABCA1 by real-time RT-PCR analysis. Statistical significance (\*,  $P < 0.05$ ) was determined by ANOVA followed by the Sidak multiple-comparisons test. Bars represent mean  $\pm$  SEM. (B) MNV4 infection decreased ABCA1 protein expression in oxLDL-treated ApoE<sup>-/-</sup> BMDM, according to western-blot analysis.

significant decrease ( $P = 0.0304$ ) in Ly6C-negative monocytes (Figure 6). No significant differences were detected in the percentages of total monocytes, macrophages and dendritic cells, macrophages, or neutrophils in the blood (Figure 6). Splenic cell subsets at 1 wk after infection did not differ between uninfected and MNV4-infected mice (data not shown). Unexpectedly, atherosclerotic lesions did not differ in size between any of the groups (Figure 7), nor did Mac2 staining reveal any intergroup differences in the percentage of macrophages within the lesion (data not shown). However, the atherosclerotic lesions of the uninfected control group were larger and varied more widely in this second study than in the first study (Figure 4 A, study 1:  $23,695 \pm 3249 \mu\text{m}^2$ ; study 2:  $33,945 \pm 3877 \mu\text{m}^2$ ), suggesting that the disease phenotype can vary even when experiments are performed under similar conditions.

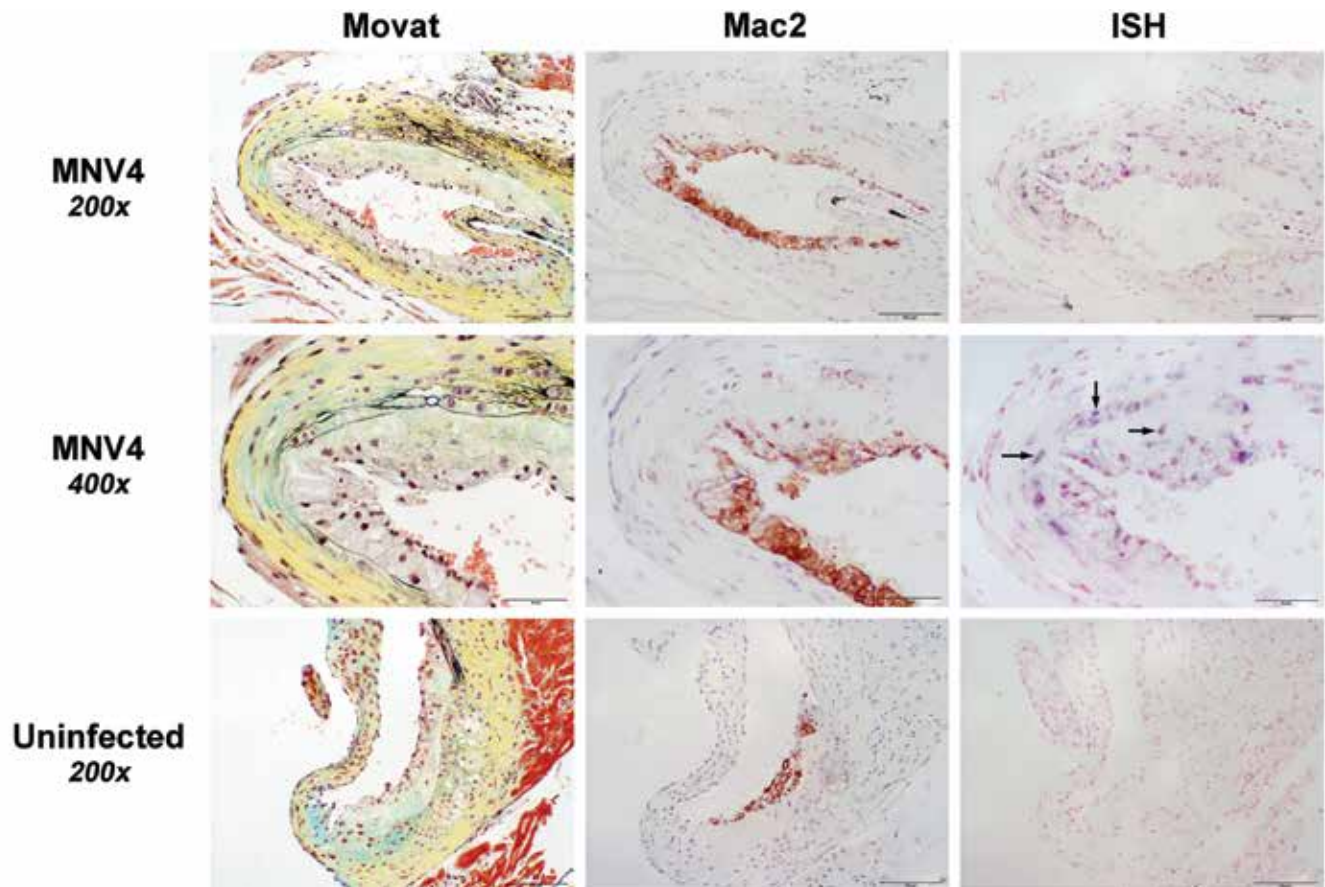
**Effect of MNV4 in ApoE<sup>-/-</sup> mice fed a high-fat, high-cholesterol diet.** Although ApoE<sup>-/-</sup> mice develop atherosclerosis when fed normal chow, ApoE<sup>-/-</sup> mice often are fed a western-type diet (high fat, high cholesterol) to accelerate disease development.<sup>15,23</sup> To determine whether MNV4 infection has an effect on a more rapidly developing and severe model of atherosclerosis, we fed male ApoE<sup>-/-</sup> mice a western-type diet for 3 wk (beginning at 4 wk of age) and then infected them with MNV4 at 7 wk of age. Mice were maintained on the western-type diet until plaque analysis was performed at 15 wk of age. In addition, we evaluated various tissues, including the aorta, from a different subset of mice by RT-PCR analysis at 1 and 2 wk after infection to determine whether MNV4 localizes to sites of active atherosclerotic lesion development. As expected, MNV4 RNA was detected in



**Figure 4.** MNV4 increases atherosclerotic plaque lesion size in ApoE<sup>-/-</sup> mice maintained on a normal chow diet. ApoE<sup>-/-</sup> mice fed a normal chow diet were infected with MNV4 at 12 wk old and evaluated at 21 wk old for the development of atherosclerosis. Lesion area in the aortic sinus was assessed by Movat pentachrome staining and by Mac2 immunohistochemical staining. (A) Significantly (\*,  $P < 0.05$ ) larger lesion areas were found in MNV4-infected mice compared with uninfected controls. (B) Percentage of macrophage area within the lesions were not different in MNV4-infected mice as compared with uninfected controls. Bars represent mean  $\pm$  SEM.

100% of the mesenteric lymph nodes (12 of 12 mice) and in a high percentage of spleens (10 of 12 mice) at both 1 and 2 wk after infection. Notably, MNV4 RNA was detected in the aortic arch in 50% (6 mice, 1 wk after infection) to 66% (8 mice, 2 wk after infection) of samples and in the descending aorta in 58% of samples (7 mice, both time points) but was not detected in bone marrow. These results indicate that MNV4 can localize to sites where atherosclerotic lesions are developing shortly after infection. However, plaque analysis at 8 wk after infection revealed no differences in atherosclerotic lesion size or percentage macrophage area in the lesion between MNV4-infected mice and uninfected controls (Figure 8 A and B). As expected, uninfected ApoE<sup>-/-</sup> mice fed the western-type diet (Figure 8 A) developed much larger atherosclerotic lesions than did uninfected ApoE<sup>-/-</sup> mice fed a normal chow diet in the previous studies (Figures 4 A and 7). Serum concentrations of 22 cytokines and chemokines at study endpoint did not differ between MNV4-infected ApoE<sup>-/-</sup> mice fed a western-type diet and uninfected controls (data not shown).

**Effect of MNV4 viral strain on atherosclerosis in ApoE<sup>-/-</sup> mice.** In our in vivo studies, MNV4 had a variable effect on atherosclerosis in ApoE<sup>-/-</sup> mice fed a normal chow diet. In study 1, MNV4



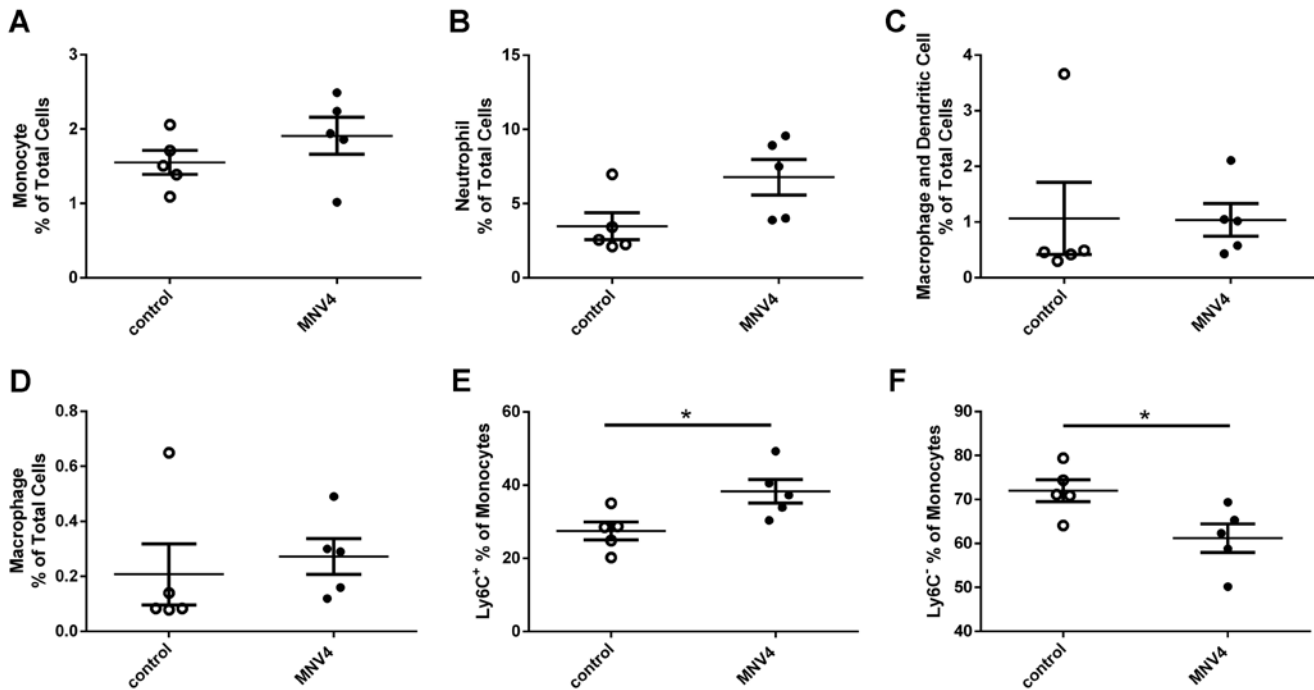
**Figure 5.** Histopathology of aortic sinus sections in ApoE<sup>-/-</sup> mice maintained on a normal chow diet and infected with MNV4. ApoE<sup>-/-</sup> mice fed a normal chow diet were infected with MNV4 at 12 wk old and evaluated at 21 wk old for the development of atherosclerosis. Lesion area in the aortic sinus was assessed by Movat pentachrome staining, macrophage area within lesions by Mac2 immunohistochemical staining (hematoxylin counterstain), and MNV4 RNA by in situ hybridization (nuclear fast red counterstain). Cells within atherosclerotic lesions were positive (purple) for MNV4 RNA by in situ hybridization (arrows); no positive staining was found in uninfected sections. Magnification, 200 $\times$  and 400 $\times$ .

infection increased plaque size by 1.7 times compared with that in uninfected controls (Figure 4 A), whereas in study 2, MNV4 infection did not alter atherosclerosis (Figure 7). When attempting to account for the different findings between the 2 studies, we realized that each study had been performed with a different preparation of MNV4. The passage number of the virus preparation used in study 1 (Figures 4 and 5) and for the in vitro experiments (Figures 1 through 3) was unknown. Therefore, to obtain a known, low-passage MNV4 stock, we generated a new preparation from an original frozen virus stock.<sup>34</sup> This low-passage (passage 7) MNV4 preparation was used for study 2 and all subsequent studies (Figures 6 through 8). Because RNA viruses have a high mutation rate,<sup>16,82</sup> the in vivo potential of the 2 viruses might have differed due to viral mutation. Nucleotide sequence comparison between positions 1664 and 2635 of the 2 viruses we used revealed 2 nucleotide differences, both corresponding to amino acid differences, at positions 2209 (T and C, valine and alanine) and 2230 (G and T, serine and isoleucine). When ApoE<sup>-/-</sup> BMDM were infected with the MNV4 preparation used in study 2, the expression of CD36 protein was increased and that of ABCA1 protein was decreased (data not shown), similar to the results obtained with the MNV4 used in study 1. However, MNV induction of cytokines in ApoE<sup>-/-</sup> BMDM was different between the 2 MNV4 preparations. Notably, the MNV4 preparation used in study 1

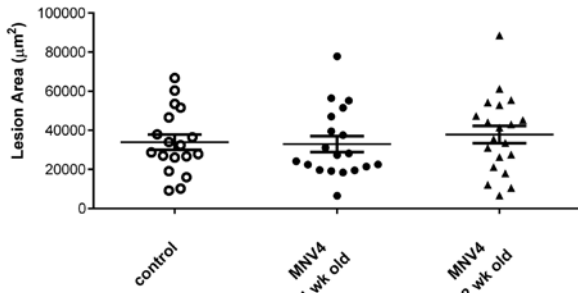
increased gene expression of iNOS, MCP1, IL1 $\beta$ , IL6, IFN $\beta$ , and TNF $\alpha$  in ApoE<sup>-/-</sup> BMDM to a much greater extent than did the MNV4 preparation used in study 2 (Figure 9). This difference in the in vitro effect of the 2 virus preparations on macrophages—cells that are pivotal for development of atherosclerosis—might explain the observed differences regarding the effects of MNV on the development of atherosclerosis in ApoE<sup>-/-</sup> mice.

## Discussion

The development of atherosclerosis involves interactions among various immune cells, including macrophages, T cells, B cells, mast cells, and dendritic cells.<sup>26</sup> Among these, monocytes and macrophages are the most intensely studied in association with the development and progression of atherosclerosis and are intimately involved in lipid accumulation in the arterial walls.<sup>26,52,73</sup> Recent studies show that different subtypes of macrophages, which may arise from different subsets of monocytes, reside in atherosclerotic lesions.<sup>24,73</sup> Macrophages are heterogeneous cells with both proinflammatory (type I, classically activated) and antiinflammatory (type II, alternatively activated) properties. These 2 subtypes may play opposing roles in the progression of atherosclerosis. MNV is an infectious agent prevalent in research mouse colonies<sup>35,66</sup> and has been shown to have a predilection to



**Figure 6.** Ly6C monocyte populations are altered in the peripheral blood of MNV4-infected ApoE<sup>-/-</sup> mice. A significantly (\*,  $P < 0.05$ ) increased percentage of Ly6C-positive monocytes with a corresponding decrease in Ly6C negative monocytes was found in the peripheral blood 1 wk after the infection of ApoE<sup>-/-</sup> mice with MNV4. Peripheral blood cells were gated on forward and side scatter and further separated into Lin<sup>-</sup> (Lin: NK1.1, CD90.2, CD49b, B220, Ly6G, and Ter119)CD11b<sup>+</sup> cells and Lin<sup>+</sup>CD11b<sup>+</sup> cells. These populations subsequently were separated on the basis of F4/80, class II, and CD11c expression to provide populations that represented (A) monocytes (Lin<sup>-</sup>CD11b<sup>+</sup>F4/80<sup>+</sup>class II<sup>-</sup>CD11c<sup>-</sup>), (B) neutrophils (Lin<sup>+</sup>CD11b<sup>+</sup>F4/80<sup>+</sup>class II<sup>-</sup>CD11c<sup>-</sup>), (C) macrophages and dendritic cells (Lin<sup>-</sup>CD11b<sup>+</sup>class II<sup>+</sup>), and (D) macrophages (Lin<sup>-</sup>CD11b<sup>+</sup>F4/80<sup>+</sup>class II<sup>+</sup>). Monocytes were further characterized according to Ly6C expression (E, Ly6C<sup>+</sup>; F, Ly6C<sup>-</sup>). Bars represent mean  $\pm$  SEM.



**Figure 7.** Atherosclerotic plaque development in ApoE<sup>-/-</sup> mice maintained on a normal chow diet and infected with MNV4 either at 4 or 12 wk of age. Mice were evaluated at 21 wk old. No differences in atherosclerotic lesion area were found between any of the groups. Bars represent mean  $\pm$  SEM.

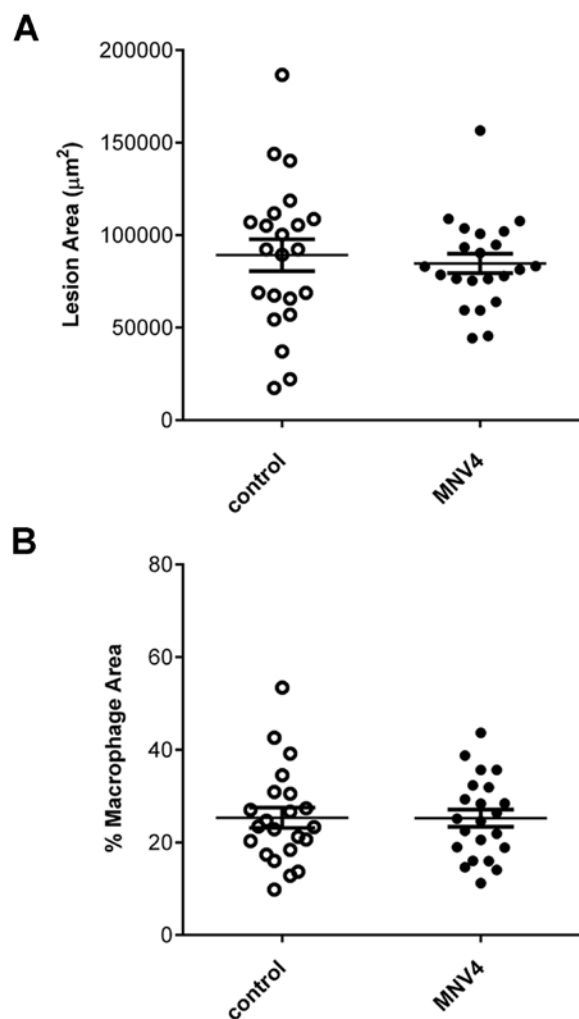
infect macrophages and dendritic cells.<sup>81</sup> Because macrophages are a key cellular component contributing to atherosclerosis, we hypothesized that MNV infection in laboratory mouse models of atherosclerosis might alter these disease models. In support of this theory, our laboratory recently reported that MNV infection, specifically with MNV4, increased atherosclerotic lesion size and macrophage content in LDLR<sup>-/-</sup> mice fed a western-type (high-fat, high-cholesterol) diet.<sup>62</sup> Our findings are consistent with the notion that an infectious agent may play a role in the development of atherosclerosis similar to *Chlamydia pneumoniae*, HIV, cytomegalovirus, and influenza virus, all of which have been implicated

to exacerbate atherosclerotic disease in humans and some mouse models.<sup>8,25,69,71,76</sup>

How a viral agent, such as MNV, either by a direct or an indirect mechanism, alters plaque development remains an open question.<sup>69</sup> One potential explanation may be that infection alters the macrophages that are pivotal to lesion development. We show here that in BMDM from ApoE<sup>-/-</sup> mice, MNV4 infection alone increased the mRNA expression of iNOS, MCP1, IL1 $\beta$ , IL6, IFN $\beta$ , and TNF $\alpha$  and, in the presence of oxLDL, MNV4 infection increased the mRNA expression of iNOS, MCP1, and IL6. The production of nitric oxide by iNOS in classically activated proinflammatory M1 macrophages has been suggested to be proatherogenic in humans,<sup>41,56,58,70,80</sup> and double-knockout mice deficient in ApoE and iNOS had decreased atherosclerotic lesion area when compared with ApoE<sup>-/-</sup> mice.<sup>40</sup> MCP1, IL1 $\beta$ , IL6, IFN $\beta$ , and TNF $\alpha$  are proinflammatory cytokines and chemokines that contribute to the development of atherosclerosis.<sup>1,26,39,45,70</sup> Our experimental data indicate that BMDM from ApoE<sup>-/-</sup> mice have altered proinflammatory responses as a result of MNV4 infection, either alone or in the presence of oxLDL.

To determine whether MNV4 infection might alter cholesterol accumulation within macrophages, we evaluated the expression of CD36 and ABCA1. CD36 is a scavenger receptor on the surface of macrophages associated with lipid uptake, foam cell formation, and the development of atherosclerotic lesions.<sup>13,20,85</sup> MNV4 infection both without and with the presence of oxLDL increased the cell-surface expression of CD36 protein, suggesting that MNV4-infected macrophages may increase their uptake





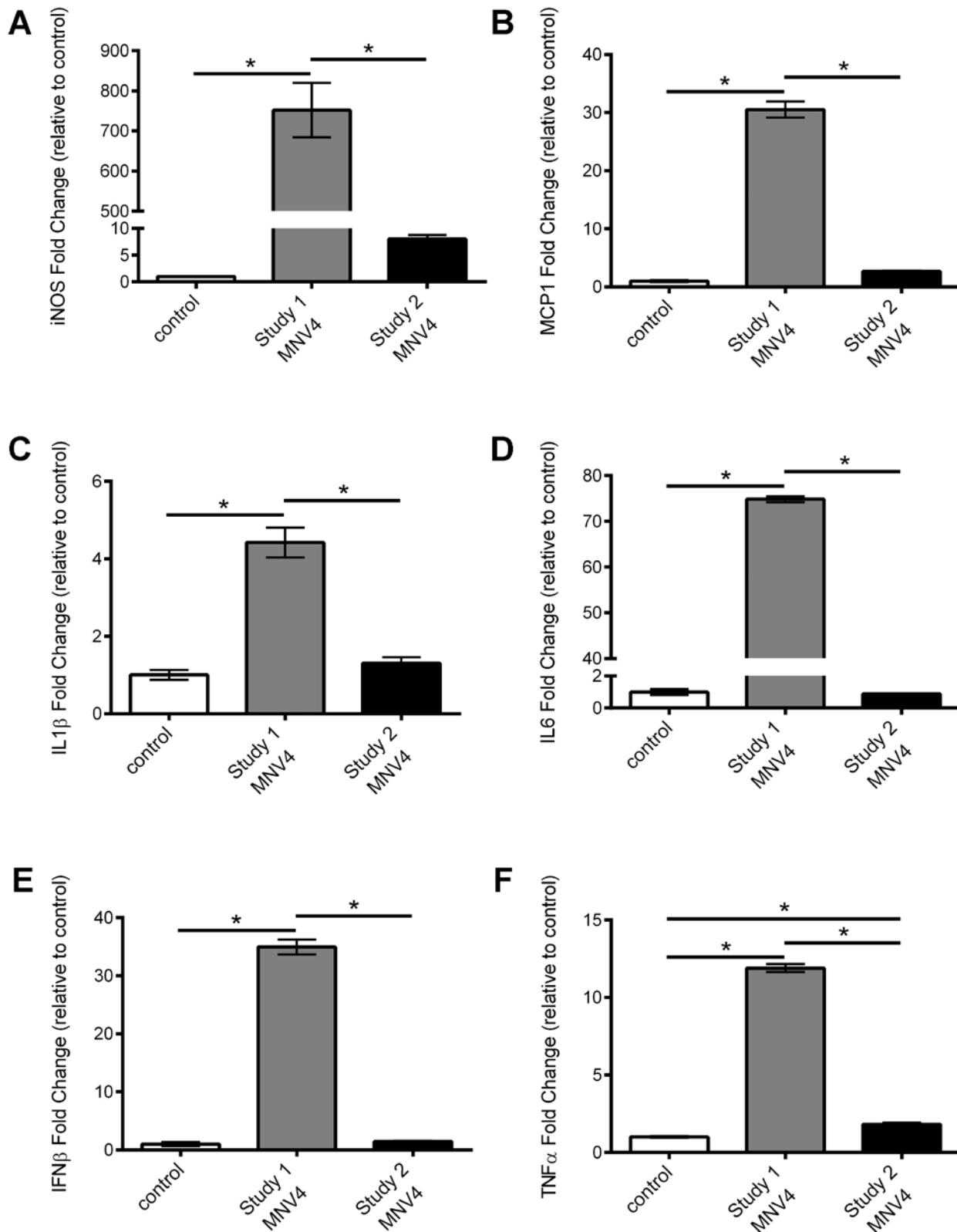
**Figure 8.** MNV4 infection does not alter atherosclerosis in ApoE<sup>-/-</sup> mice maintained on a western-type (high-fat, high-cholesterol) diet. ApoE<sup>-/-</sup> mice were fed a high-fat, high-cholesterol diet beginning at 4 wk of age, infected with MNV4 at 7 wk old, and then evaluated at 15 wk old for the development of atherosclerosis. (A) Atherosclerotic lesion areas did not differ between MNV4-infected mice and uninfected controls. (B) The percentage of macrophage area within the lesions did not differ between MNV4-infected mice and uninfected controls. Bars represent mean  $\pm$  SEM.

of cholesterol. ABCA1 is an ATP-binding cassette transporter protein that plays an important role in cholesterol efflux from macrophages and foam cells.<sup>41,78,83</sup> The activation of inflammatory signals, such as through activation of the Toll-like receptors TLR3 or TLR4 or by infection with infectious agents such as *Escherichia coli* or influenza A virus, can block liver X receptor-dependent pathways, including ABCA1, thereby decreasing cholesterol efflux.<sup>11,41,84</sup> This crosstalk between Toll-like receptors and liver X receptor is mediated by IFN regulatory factor 3,<sup>11,84</sup> and both TLR3 and IFN regulatory factor 3 reportedly play important roles during MNV infection,<sup>49,74</sup> thus providing a potential mechanistic link between MNV infection and ABCA1. Indeed, we found that MNV4 infection of BMDM from ApoE<sup>-/-</sup> mice in the presence of oxLDL decreased the protein expression of ABCA1, suggesting that cholesterol efflux is decreased in

MNV4-infected macrophages. Similarly, HIV has been reported to inhibit ABCA1-mediated cholesterol efflux in macrophages to increase its viral replication, as HIV infection promotes foam cell formation and increases the risk of atherosclerosis in HIV-infected people.<sup>7,14,53,54</sup> Interestingly, the cellular cholesterol pathway may be important for norovirus replication,<sup>12</sup> and cellular plasma membrane cholesterol is required for entry into and infection of macrophages via endocytosis by MNV1,<sup>22,64</sup> thus suggesting a link between cholesterol and cholesterol transport pathways and norovirus infection.

Our in vitro data suggesting that MNV4 infection modifies both inflammatory and cholesterol-transport pathways led us to hypothesize that these pathways might likewise be modified within the macrophages of atherosclerotic plaques in vivo. In the first in vivo study, in which ApoE<sup>-/-</sup> mice fed a normal chow diet and then infected with MNV4 at 12 wk old had significantly larger aortic-sinus lesion sizes than did uninfected control mice. In situ hybridization for MNV4 within atherosclerotic lesions as well as RT-PCR analysis for MNV4 in aortic tissues were positive in some mice, confirming the presence of detectable virus at sites of atherosclerotic plaque development. These findings suggest that a direct effect of the virus to induce disease exacerbation within the lesion itself is possible. A direct effect on atherosclerosis has been reported for other infectious agents, some of which, such as influenza and cytomegalovirus, have been found within the atherosclerotic plaque itself.<sup>25,68,69,76,77</sup> However, although MNV4 was found in aortic plaques, the possibility that indirect mechanisms may also have a role cannot be ruled out entirely, given that MNV causes persistent and systemic infections in mice.<sup>34</sup> In addition, MNV4 infection increased the percentage of Ly6C-positive monocytes in the blood 1 wk after infection, suggesting another possible mechanism by which infection may alter disease. Ly6C-positive monocytes have been associated with a proinflammatory response through enhanced recruitment of monocytes into atherosclerotic lesions, differentiation into macrophages, and the formation of foam cells.<sup>67,72,73</sup>

We found that the effect of MNV4 infection on atherosclerotic lesion development in ApoE<sup>-/-</sup> mice maintained on a normal chow diet varied between in vivo studies 1 and 2. This variable result could be due to one or more of several possible explanations. One explanation may be fluctuating numbers of MNV4-infected macrophages and monocytes in plaques, thus variably influencing lesion progression, especially if direct infection of plaques is the major contributor to disease development. In support of this notion, the aortas of only a subset of infected mice were positive for MNV4 by RT-PCR analysis, even though all animals were confirmed infected with MNV by fecal RT-PCR assay. This variation in detectable virus in the aorta, although possibly due to the sensitivity of the detection method, might be a true indicator of the variability and potentially fluctuating levels of MNV4-infected monocytes and macrophages within plaques at any given time. A second alternative explanation of the variable effect of MNV4 infection on atherosclerosis is that any potential influence of infection on disease development might have been masked by the larger variation in plaque lesion size in uninfected control mice in study 2 compared with those in study 1. In humans, the development of atherosclerosis has been proposed to occur intermittently, with times of idleness intermixed with periods of rapid progression, rather than in a linear fashion;<sup>44</sup> this nonlinear disease progression may



**Figure 9.** Inflammatory gene expression is differentially altered in ApoE<sup>-/-</sup> BMDM infected with MNV4 from different viral preparations. Inflammatory gene expression was measured by real-time RT-PCR analysis in ApoE<sup>-/-</sup> BMDM after infection (multiplicity of infection, 0.2; triplicate wells per group) with MNV4 from 2 different virus preparations for 24 h. Increases in the gene expression of (A) iNOS, (B) MCP1, (C) IL1 $\beta$ , (D) IL6, (E) IFN $\beta$ , and (F) TNF $\alpha$  were dependent on which MNV4 virus preparation (that is, that for in vivo study 1 compared with study 2) was used for infection. Statistical significance (\*,  $P < 0.05$ ) was determined by ANOVA followed by the Sidak multiple-comparisons test between all groups. Bars represent mean  $\pm$  SEM.

account for the larger variation in plaque lesion area in study 2 as compared with study 1. Therefore, it may be difficult to consistently detect MNV4-induced alterations in atherosclerosis, given this variability in uninfected controls. Finally, a more compelling reason to explain the different results obtained between studies 1 and 2 is potential differences between the 2 virus preparations used for each study. RNA viruses, including MNV, have a high mutation rate due to their lack of a 3'-to-5' exonuclease proofreading activity in their RNA-dependent RNA polymerase;<sup>16,82</sup> consequently mutations in MNV4 can occur during viral propagation. In support of this explanation, our comparison of a 972-bp segment of the 2 viruses revealed 2 single-nucleotide differences, both of which resulted in amino acid changes. Notably, infection with the 2 different MNV4 viruses produced vastly different cytokine and chemokine gene expression responses in ApoE<sup>-/-</sup> BMDM. These results indicate that there are indeed differences in the 2 MNV4 virus stocks we used in the studies, and perhaps these differences contributed to the variable effect on atherosclerosis seen in vivo. In support of this idea, others have reported that different MNV strains, some with as little as a single amino acid substitution, differ markedly in their persistence, replication, or virulence in infected mice.<sup>3,37,55,61,75,86</sup> Regardless of the reason, these data highlight the variable nature of whether changes in the severity of atherosclerosis will be observed as a result of MNV infection and are noteworthy given that the effect of infection may be unpredictably evident each time infection occurs.

In 2009, a workshop sponsored jointly by the NIH, National Center for Research Resources, and National Institute on Aging, entitled "Detection, Impact, and Control of Specific Pathogens in Animal Resources Facilities" listed as one of its specific goals the "identification of current gaps in scientific knowledge such as...the pathobiology of recently identified pathogens such as norovirus infection in mice."<sup>48,59</sup> This workshop led to subsequent funding opportunities.<sup>60</sup> Our laboratory contributed to this goal by continuing our efforts to evaluate the effect of MNV infection on mouse models of atherosclerosis. We report that MNV4 infection in ApoE<sup>-/-</sup> mice variably alters the ApoE<sup>-/-</sup> mouse model of atherosclerosis and should be considered when performing experiments in MNV endemic research mouse colonies. Our laboratory has also previously evaluated MNV infection in other chronic disease models, such as those for inflammatory bowel disease. We previously showed that MNV can alter inflammatory bowel disease in Mdr1a<sup>-/-</sup> mice (FVB.129P2-*Abcb1a*<sup>tm1Bor</sup>)<sup>43</sup> but this confounding effect was not demonstrated in Smad3<sup>-/-</sup> (129-*Smad3*<sup>tm/Par</sup>/J) mice<sup>42</sup> or in IL10<sup>-/-</sup> (B6.129P2-*Il10*<sup>tm1Cgn</sup>/J) mice.<sup>32</sup> Similarly, other laboratories have corroborated the variable influence of MNV infection on mouse models, with some describing little or no effect,<sup>2,29,30</sup> and others reporting altered disease phenotypes as a result of MNV infection.<sup>4,9,37</sup> Collectively, these studies highlight the variable effect that MNV infection can have on mouse models of inflammatory diseases. We therefore propose that performing experiments in MNV-free mouse colonies is warranted due to the variable and sometimes unpredictable confounding effect this virus can have on the development of inflammatory diseases.

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